Exploring and exploiting natural variation in the wings of a predatory ladybird beetle for biological control

Suzanne Lommen

Lommen, Suzanne T. E.

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Cover: the front page shows a part of a flight wing of the two-spot ladybird beetle, *Adalia bipunctata*, the species of ladybird beetle studied in this thesis. The pattern is formed by the veins and pigments. The back page depicts a part of a leaf of a lime tree, one of the plant species that was treated with wingless forms of this ladybird for the biological control of aphids.

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Ter herinnering aan | *In memory of*

Hideki Ueno

die dit onderzoek begon | *who initiated this research*

Michael Majerus

groot evolutionair bioloog | *an important evolutionary biologist*

met een passie voor lieveheersbeestjes | *fascinated by ladybirds*

en bovenal | *and mainly*

Wouter

omdat je wilde dat ik door zou gaan | *because you wanted me to carry on*

Contents

Collecting ladybirds in Utrecht with Kees Koops

General introduction

Suzanne T.E. Lommen

Aphids form major pests on many crops and trees (Blackman and Eastop 1984; 1994). Predatory ladybird beetles are amongst their most important natural enemies, but their current use in the biological control of aphids is limited (Michaud 2012). One reason is that they tend to fly away from the plants soon after release. With this thesis, I aim to gain knowledge about natural variation in wing length in a Dutch population of the predatory two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), and to use this to improve biological control of aphids.

In this chapter, I initially introduce some important concepts in biology related to fundamental questions about natural variation, because these theories are the framework of the following experimental work. I then provide an outline of the spectacular diversity in insect wings, along with some related theories that can may explain this variation. Consequently, I give an introduction on biological control by ladybirds. After that, I narrow the introduction to the study system of *A. bipunctata*, including its biology, variability in wing morphology, and its potential use in biological control of aphids. Finally, I provide an overview of the following thesis chapters.

Natural variation and concepts in biology

Natural variation in living organisms has always fascinated humans. Ancient societies began to study this variation, and also to exploit it by practising selective breeding of animals and plants. One of the major challenges in biological science is to explain this variation. Natural variation inspired Darwin to develop his evolutionary theory on the origin of species, identifying **natural selection** of traits as the driving force (Darwin 1859) (Box 1). This concept has remained the basis for the field of evolutionary biology although it has been modified since by incorporating new insights. In the 20th century, it was recognized that **genes are the unit of inheritance**, and the Modern Synthesis of evolutionary biology was developed, based on the principles of population genetics (Mayr 2002). Since molecular biology expanded our knowledge, it has become clear that the Modern Synthesis is too simplified. There are alternative explanations for the maintenance of genetic variants, including **mutation bias**, **gene flow** resulting from migration or horizontal gene transfer, stochastic processes such as **genetic drift**, and **hitchhiking** (Maki 2002; Gillespie 2006; Chan et al. 2009; Koonin 2009; Boto 2010) (Box 1). Recent advantance in genome biology paradoxically provide accumulating evidence for **non-genetic inheritance** of traits (reviewed by Danchin et al. 2011). These phenomena should be integrated into the existing paradigm. In this thesis, established evolutionary concepts form the framework to discuss the existence of wing length variation in *A. bipunctata*.

Mayr made an important contribution to the field of biology by distinguishing **proximate** and **ultimate** explanations for biological systems (1961). Proximate explanations concern **mechanisms**

Box 1. Concepts in genetics and evolutionary biology

(Based on Gillespie 2001; Maki 2002; Morjan and Rieseberg 2004; Futuyma 2005; Lynch 2007; Danchin et al. 2011)

Heredity

Heredity is the transmission of traits from parent to offspring. A trait is a characteristic of an organism (e.g. elytral colouration in a two-spot ladybird beetle, see Fig 1 in chapter 4). Modes of transmission can be divided into genetic and non-genetic.

- **Genetic heredity**: genes are the unit of inheritance. The Modern Synthesis of Evolution is based on the principle of genetic inheritance. Some related terms:
  - **gene**: a piece of DNA that is the physical and functional unit of heredity, underlying a trait
  - **allele**: the alternative form of a gene for a character (e.g. the allele for the melanic colour, and the allele for the typica colour)
  - **locus**: the location of a gene or DNA sequence on a chromosome (e.g. the loci for elytral colouration and winglessness are on the same chromosome)
  - **genotype/genotypic trait**: the set of genes in the DNA of an organism/the constitution of the genes of a specific trait of that organism (e.g. a ladybird with a elytral colouration genotype of one melanic and one typical allele)
  - **phenotype/phenotypic trait**: the expression of a genotype as an observable characteristic of an organism, as determined by the interaction of its genotype and environmental influences (e.g. the ladybird with one melanic and one typical allele for elytral colour has a melanic phenotype, because the melanic allele is dominant to the typical allele)
  - **genome**: the complete set of genetic material of an organism
- **Non-genetic heredity**: non-genetic information inherited across generations. Nowadays, it is recognized that this mode of transmission also contributes to the observed variation in phenotypes. Non-genetic mechanisms of inheritance include epigenetic inheritance, parental effects, ecological inheritance and cultural inheritance.

Biological evolution

Biological evolution is the change in the inherited characteristics of populations of living organisms over successive generations. Thus, evolution is the change in allele frequencies in a population. Currently, four mechanisms are recognized to drive evolution:

- **Mutation**: a heritable change in the DNA sequence in the genome of a cell. This is a source of genetic variation. Mutational bias: different mutations have different probabilities of occurring. Mutational hotspot: a part of the genome with an elevated chance for the occurrence of mutations.
- **Gene flow**: the exchange of genes between populations and between species. Gene flow can occur through different processes:
  - **migration**: the movement of individuals
  - **horizontal gene transfer**: the incorporation of genes from another organism, in a manner other than reproduction
- **Natural selection**: because of limited resources, there is competition between organisms for survival and reproduction, and genetic mutations that enhance reproduction become and remain more common in successive generations of a population than genetic variants that reproduce less well (popularly called: “survival of the fittest”). Related terms:
  - **fitness**: the ability to both survive and reproduce in a given environment (so, how well an organism is adapted to the circumstances where it lives in)
  - **adaptation**: the evolutionary process whereby an organism becomes better able to survive and reproduce in the environment it normally lives in
  - **sexual selection**: a special case of natural selection, where the preference by one sex for certain characteristics of the other sex increases mating success, and thus, reproduction, resulting in selection for these traits
- **Stochastic processes**: random processes, including:
  - **genetic drift**: allele frequencies change in a small population due to random events unrelated to the identity of the alleles themselves
  - **genetic draft** or **genetic hitchhiking**: the frequency of an allele changes because it is physically linked to another gene that is under natural selection

explaining how systems work, including the genetics, development, and physiology, whereas ultimate explanations make clear why these systems have evolved, addressing the consequences of a trait for survival and reproductive success, that is **fitness**. Mayr (1961) emphasized that these **views are complementary**. However, in recent decades it has become clear that the causes and consequences of a trait cannot always be separated, because some mechanisms contribute to both the function and the evolution of a system (Laland et al. 2011). For example, the field of evolutionary development ("evo-devo") recognizes that developmental mechanisms determine the phenotype of an organism, but can also influence the speed and direction of evolution (Brakefield 2006). Nevertheless, the "how" and "why" questions remain central in biology, and are both treated in this thesis.

Diversity in insect wings

Evolution of wings

Insects form the most species-rich group of animals, with nearly a million described species inhabiting all parts of the world (Grimaldi and Engel 2005). The evolution of flight is thought to be a key innovation for their success, increasing the range of **active dispersal,** and thereby facilitating the search for food, habitats, and mates (Wagner and Liebherr 1992). It is thought that wings appeared in a single event in the insect lineage about 400 million years ago (Engel and Grimaldi 2004). This first winged insect is thus the last common ancestor of all winged species of insects that derived from it. The ability of flight required other modifications, most importantly flight muscles to move and steer the wings.

As a result of evolution, wings are **extremely diverse** in the current insect fauna. They vary not only in appearance but also in function, because they have other functions than flight in many species. For example, they confer physical adjustments to the abiotic environment, and can provide and/or produce visual, olfactory, and audible signals that play a role in protection from enemies, recognition of conspecifics, and courtship behaviour (Capinera 2008).

Evolution of flightlessness and winglessness

Despite the advantages of flight, secondary **loss of flight ability** has occurred independently and repeatedly during evolution in most winged insect orders (Roff 1990). Loss of flight is often accompanied by loss of wing tissue. It is estimated that ten percent of all species contain wingless individuals (Zera and Denno 1997), but this high percentage may also be explained by an enhanced diversification after the loss of flight, due to the limited dispersal which increases the chance of genetic isolation (Roff 1994a; Ikeda et al. 2012; but see Vogler and Timmermans 2012).

In some cases species have become flightless, such that all individuals lack the ability to fly. However,

in other cases variability in flight ability is found within species ("**intraspecific variation**") (Roff 1994a). The latter species can be extremely suitable for the study of the causes and consequences of winglessness since they allow comparison between winged and wingless conspecifics. In some of these **wing polymorphic** species, the possession of functional wings is related to gender (Roff 1990), or to social castes (Abouheif 2004). Well known examples of wing dimorphism are the winged reproductive castes and the wingless worker and soldier castes in ant species, the winged males and the wingless females in fire flies and aphids, and the swarming flying migratory morphs versus the non-flying solitary morphs in desert locusts.

The question of how this polymorphism is regulated, has revealed cases of genetic regulation ("**genetic polymorphism**")(Roff 1986), and of environmental induction ("**polyphenism**") (e.g. Braendle et al. 2006). The question of why loss of flight has evolved has often been explained by the process of natural selection. In these cases, winglessness can be described as adaptive. The ability to fly involves **energetic costs** associated with the maintenance of the flight apparatus (the flight muscles and the wings), and of flight activity itself (Mole and Zera 1993; Zera and Mole 1994; Zera et al. 1994). The **resource allocation theory** states that individuals possess limited energetic resources that are allocated to competing life-history traits (Zera and Hashman, 2001). Therefore, the ability to fly typically comes at the cost of investment in other traits important in fitness, such as survival and reproduction. Indeed, the **trade-off** between flight and reproduction ("**oogenesis-flight syndrome**") is well-established for wing polymorphic insects, with numerous examples in different species of insects where the wingless morphs are more fecund (Roff 1986; Roff and Fairbairn 1991).

Flightlessness in ladybird beetles

Within the order of beetles (**Coleoptera**), the number of species comprising non-flying morphs has been estimated to exceed a thousand (Crowson 1981; Roff 1990, 1994a). Within the family of ladybird beetles (**Coccinellidae**), only a few species of ladybird beetles exhibit intraspecific variation for flight ability. Variation in flight ability results from variation in flight wing length and shape in *Rhyzobius litura* Fabricius, *R. chrysomeloides* (Herbst), and *Subcoccinella 24-punctata* (L.) (Pope 1977). In addition, variation in flight wing folding has been described for *R. litura* (Hammond 1985). The multicoloured Asian ladybird *Harmonia axyridis* Pallas exhibits quantitative variation in flight ability not associated with any changes in the wings (Tourniaire et al. 2000). Finally, rare wingless morphs with reduced flight wings and elytra are found in populations of the two-spot ladybird beetle *Adalia bipunctata* (Marples et al. 1993). In these ladybird species, a potential adaptive value of limited dispersal by flight has not been found (Ueno et al. 2004). However, intraspecific variation in flight ability of the **predatory** species *H. axyridis* and *A. bipunctata* is of great interest with respect to their use as **biological control agents** of aphids.

Biological control by ladybird beetles

In biological control, **natural enemies** of pests are used to control them. Van Lenteren (2012) argues that biological control is generally the most environmentally safe and economically profitable pest management method. Aphid-eating ("**aphidophagous**") ladybirds (Fig. 1) are used for the biological control of aphids.

Population dynamics theory based on **predator-prey interactions** predicts that naturally occurring aphidophagous ladybirds are not able to control aphid populations effectively in nature and agro-ecosystems (Dixon 2000). First, because females will never deplete a site with aphids where they lay their eggs since their offspring would starve. Secondly, because the development and reproduction of these ladybirds takes much longer than that of their prey, and as a result, they cannot keep up with the population growth of aphids. Several types of professional biological control are distinguished (see Box 2 for definitions and details). With respect to **conservation biological control** in agro-ecosystems, the contribution of aphidophagous ladybirds varies largely. They will never attain long-term suppression of aphids, but may well reduce within-season aphid densities by reduction of peak aphid densities (Obrycki et al. 2009).

Ladybirds have a long history in **classical biological control** programs. However, none of the aphidophagous species introduced in exotic geographical areas were considered to be successful by Dixon (2000).

Aphidophagous ladybirds are also used in **augmentative control**. However, to achieve effective aphid control, they should be introduced into the system well before aphid populations start to build up, which is earlier than the moment that they would naturally arrive, and multiple introductions may be required (Dixon 2000). There is little empirical and theoretical support for the success of ladybird beetles in **inoculative augmentation** (Obrycki and Kring 1998; Dixon 2000). In contrast, there are several examples where ladybirds were successfully applied by means of **inundative augmentation** (Powell and Pell 2007). The ladybird *Harmonia axyridis* Pallas has been used for such control and has been considered one of the 25 most commonly used biological control agents in Europe (Van Lenteren 2003a), until this species was recently banned from commercial use in Europe for being exotic and invasive (Brown et al. 2008; Van Lenteren et al. 2008).

Cost-effective inundation of ladybirds is, however, complicated for several reasons. **Commercial mass-rearing** of ladybirds is costly because of their predacious and cannibalistic life style (Van Lenteren 1988). To achieve effective control, the **timing of release** is essential, which requires both intensive scouting for the pest, and knowledge of the population dynamics of the ladybirds (Michaud 2012). Finally, it is considered that the **flight ability** of adults (Fig. 2) is a major obstacle. Their tendency to fly away from the host plants (e.g. Gurney and Hussey 1970) requires repeated releases (Obrycki and Kring 1998), which is costly. Attempts to improve the efficacy of biological

control of aphids have involved **limiting their flight dispersal**. For example, Ignoffo et al. (1977) artificially trimmed wings of *Hippodamia convergens* Guérin-Méneville, which increased both their residence time and predation after release in an aphid-infested soybean field. Several researchers achieved to reduce the flight ability of *Harmonia axyridis* Pallas. In one case, chemically induced mutagenesis yielded a morph with malformed wings (Ferran et al. 1998). This strain resided longer on aphid-infested cucumbers in a greenhouse, but their high mortality and low reproduction hampered efficient mass production (Ferran et al. 1998). In two other cases, non-flying morphs of this species but with intact wings were obtained by artificial selection on reduced flight ability, with mixed effects on fitness traits (Tourniaire et al. 2000; Seko et al. 2008). Both proved successful in biological control of aphids on crops in greenhouses or the open field, including one case where they were more successful than winged conspecifics (Weissenberger et al. 1999; Seko et al. 2008; Adachi-Hagimori et al. 2011; Iguchi et al. 2012). This thesis focuses on the potential for naturally occurring flightless morphs of *A. bipunctata* to improve inundative control of aphids.

Box 2. Biological control

(Definitions and concepts from Eilenberg et al. 2001; Hajek 2004; Van Driesche et al. 2008)

Biological control or "**biocontrol**" is the use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be. The antagonists used for this purpose are natural enemies of the pests, and are called "biological control agents".
Several control strategies can be distinguished:

- **Conservation biological control**: resident populations of natural enemies are conserved or enhanced by modification of the environment or existing practices. The aim is to reduce the effect of the pest, not to yield complete control.
- **Classical biological control** (also called inoculative control, but not in this thesis)**:** the intentional introduction of an exotic biological control agent for permanent establishment and long-term pest control. This is the most widespread and successful mode of biological control. It is applied for outdoor pests that are usually invasive exotics. The agents are natural enemies of the pest species in their native range, and are introduced into the new geographic area following the pest species.
- **Augmentation**: the release of extra natural enemies at the place where the pest should be controlled.
  - **Inundation** or **mass release**: large numbers of natural enemies released for immediate and short-term pest control by these specimens. They often cannot maintain a population after release, so they have to be re-applied when the pest returns or increases again in number.
  - Seasonal **inoculation**: fewer individuals released, and reproduction at the place of release is expected, so both the generation released and their progeny contribute to control, aiming at an extended period of, but not permanent, control.

  To be cost-effective, augmentative biocontrol requires the possibility for cheap **mass-rearing** and **high efficacy** at a limited release frequency.

The use of natural enemies exotic in the area released (for classical or augmentative control) is nowadays legally restricted. To be allowed, a risk assessment and evaluation of the impact have to be passed.

When chemical and biological control methods are combined in agricultural practice, this is called **integrated pest management** (IPM).

Figure 1. A seven-spot ladybird beetle eating an aphid.
(Photo taken by Hans Smid, www.bugsinthepicture.com)

Figure 2. *Adalia bipunctata* taking off, showing the full size of the unfolded flight wings.
(Photo taken by Kjeld Olesen, www.acapixus.dk)

The study system – *Adalia bipunctata*

General biology

The two-spot ladybird beetle *Adalia bipunctata* (L.) is a **generalist predator.** It is an aphidophagous ladybird that feeds on a wide range of aphid species, which vary strongly in their suitability for ladybird development: the lime aphid *Eucallipterus tiliae* has been suggested to be one of the most suitable (Mills 1981; Kalushkov 1998; Omkar and Pervez 2005; Hodek and Evans 2012). It can also feed on some other insect species including scale insects (Majerus 1994; Omkar and Pervez 2005), other species of ladybird, and conspecifics (i.e. **cannibalism**) (Mills 1982; Agarwala and Dixon 1992). In the absence of prey, individuals have been observed to feed from pollen (Hemptinne and Desprets 1986). Because of its polyphagous feeding pattern, the ladybird can be found on a **variety of host plants**, including trees, shrubs, herbs, and agricultural crops (Mills 1979; Honěk 1985; Hoffmann et al. 1997; Trouvé et al. 1997; Tomanovic et al. 2008; Dib et al. 2010).

The species is native to parts of Europe, North America, and north and central Asia. In Europe, it is **univoltine** or **multivoltine**, with respectively one, or two to four generations during the spring and summer (Brakefield 1984a; Hemptinne and Naisse 1987). In autumn, the adult beetles aggregate at cracks in trees and houses where they stay in dormancy ("**diapause**") until the next spring, when they migrate to host plants with aphids if they overwintered elsewhere (Hodek 1973; Brakefield 1984a).

The ladybird has a **holometabolous** life cycle, including eggs, and four wingless larval instars before pupation (Box 3). The adults are winged and can disperse by flight (Fig. 2). They consist of **diploid** males and females (Smith 1953) that reproduce sexually. They are highly **promiscuous** (Haddrill et al. 2008). After mating, females store the sperm inside a special organ (Hodek 1973; Arnaud et al. 2003), and they can lay fertilized eggs until shortly before the end of their lives (De Jong et al. 1998).

The female's searching activity is mainly focussed on localising aphids, because she deposits her eggs (**"oviposition"**) where her offspring will find sufficient food (Wratten 1973; Hemptinne et al. 1992; 1996; Dixon 2000). The males mainly search for female mating partners (Hemptinne et al. 1996). Since aphid colonies fluctuate widely in density and only persist on host plants for a limited period (Dixon 1998), the adult ladybirds shift between host plants with sufficient aphids, and can be found on different species of plants throughout the season (Brakefield 1984a). However, even if aphids are abundant, adult females cease to oviposit in a patch when they detect the presence of conspecific larvae by the species-specific tracks these leave on the leaf surfaces (Hemptinne et al. 1992; Doumbia et al. 1998). This oviposition behaviour can be explained by the risk of cannibalism of the eggs by conspecific larvae (Martini et al. 2009).

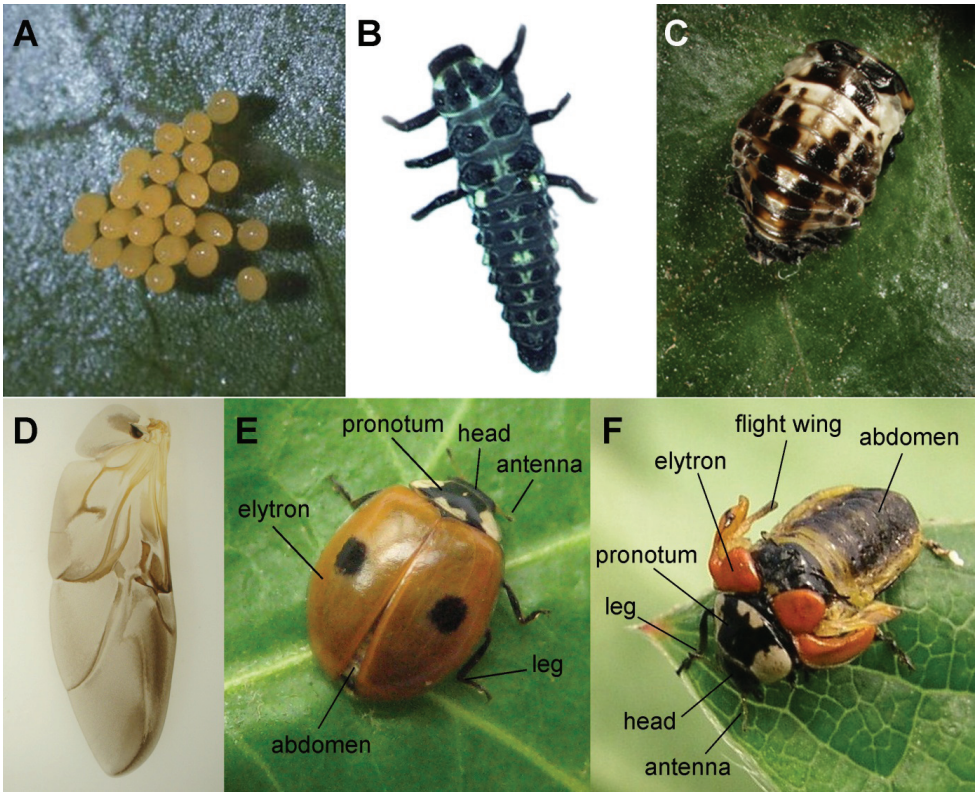
Morphological variation in the wings

Only adult beetles possess functional wings. The two hind wings of ladybirds are used to fly. The two fore wings have evolved into sclerotized, hard structures typical of beetles, and are called "**elytra**" (see Box 3 for body plan). They provide *A. bipunctata* with protection to the **flight wings** which are folded underneath, and to the soft dorsal surface of the abdomen that is typically entirely covered by the elytra when the beetle is at rest. In addition, the elytra play a role in thermoregulation (De Jong et al. 1996), mating behaviour (Hemptinne et al. 1998), and their spotted colour pattern is thought to act as a warning colouration for predators signalling unpalatability (Majerus 1994). Indeed, *A. bipunctata* contains and excretes chemical defence compounds deterring some birds (Marples et al. 1989). In other ladybirds it has been shown that predator deterrance could be attributed to the colour pattern (Marples et al. 1994), and specifically the spots in this pattern (Dolenska et al. 2009), in combination with the taste of the ladybird (Marples et al. 1994), but also the general characteristic shape of the elytra (Dolenska et al. 2009).

Adalia bipunctata exhibits intraspecific natural variation in wing morphology. The **colour polymorphism** is one of the most thoroughly studied by evolutionary biologists (e.g. Lusis 1961). Hundreds of different colour morphs have been described, but three dominate in nature: the **typical**, non-melanic morph which has red elytra with two black spots and a black pronotum with two white spots (typica), and the **melanic** morphs with an entirely black pronotum and black elytra with either four (quadrimaculata), or six (sexpustulata) red spots (Brakefield 1984a; Majerus 1998) (see Fig. 1 in chapter 4 on page 61). The trait is under genetic control (Majerus 1994). Wild populations can consist of a mixture of these morphs, but there is temporal and geographic variation in the frequencies of melanic individuals that has been associated with climate (Creed 1975; Scali and Creed 1975; Bengtson and Hagen 1977; Brakefield and de Jong 2011). Typical and melanic colour morphs also differ in their thermal properties and their activity (De Jong et al. 1996), and they are regularly observed to mate non-randomly with respect to this colouration (Majerus et al. 1982; Brakefield 1984c; Kearns et al. 1990; Zakharov and Goryacheva 1998).

Another type of striking morphological variation concerns the length of the wings: **wingless morphs** with incomplete, shorter wings occur in the wild (Majerus 1994). Such morphs are rare, but have been recorded for at least one population in The Netherlands (Marples et al. 1993), and five in the United Kingdom (M.E.N. Majerus, personal communication). In the most recent two decades, some studies have been undertaken on this phenomenon in the Dutch population. Here, winglessness is determined by one major, **single locus** with the wild-type **winged allele dominant** over the wingless one (Marples et al. 1993). So far, **no fitness advantage** has been found for the wingless morphs or the wingless allele: the wild type is favoured with respect to several life history traits, and no heterozygous advantage has been detected (Ueno et al. 2004). Therefore, the potential adaptive value of winglessness in *A. bipunctata* in nature, if any, is unknown.

Box 3. From egg to adult: development and adult body plan of *A. bipunctata*



A) A clutch of eggs. B) A fourth instar larva. C) A pupa. D) A wild-type flight wing. E) A wild-type adult. Several body parts are indicated. Not all six legs are visible, and the flight wings are folded underneath the elytra. F) A "wingless" adult with truncated elytra (fore wings) and flight wings (hind wings).

Biological control by *A. bipunctata*

The role of *A. bipunctata* in conservation biological control remains unclear. Experiments confirm that the complex of natural enemies naturally present in organic apple orchards, including *A. bipunctata*, does have a negative effect on the abundance of the rosy apple aphid, *Dysaphis plantaginea* Passerini, but was not able to prevent damage (Dib et al. 2010). The abundance and efficacy of natural enemies in this system was enhanced by the creation of specific weed strips in between the trees, a form of conservational control (Wyss 1995). However, in such studies, the role of *A. bipunctata* cannot be disentangled from that of other species.

Adalia bipunctata is being used in augmentative control of aphids. Europe is the major market

area of biological control agents for augmentation, and here *A. bipunctata* has been commercially available for fifteen years (Van Lenteren 2012). Its importance has increased since the highly invasive nature of the exotic ladybird beetle *H. axyridis* has been recognized (Brown et al. 2008; Roy and Wajnberg 2008; Van Lenteren et al. 2008). The use of this Asian species was banned by legal means several years ago. In addition, several other characteristics make *A. bipunctata* a suitable species for this type of biological control (see Box 2 for requirements of natural enemies for augmentation). It has a wide host range (Mills 1981), is active in a wide range of temperatures (Jalali et al. 2010) including relatively cold conditions (Wyss et al. 1999a), can be stored in the cool to expand its period of availability (Hämäläinen and Markkula 1977), can be reared year-round without dormancy (Hämäläinen 1976), and performs well on a diet of alternative food (De Clercq et al. 2005; Bonte et al. 2010).

The ladybird is mainly applied against aphid pests in **greenhouses** and on **urban trees**. The few studies evaluating the effect of releasing *A. bipunctata* prove that inundative control can indeed be successful. Hämäläinen (1977, 1980) showed this for several greenhouse crops. Inundative releases of *A. bipunctata* in an orchard system also demonstrate that larvae were indeed able to prevent the rosy apple aphid from building up to large populations. This resulted in a 20-fold lower abundance, provided the release was early in the season, and bad weather did not obstruct survival of the beetles (Wyss et al. 1999a; 1999b).

Despite these positive results in inundative aphid control, the use of *A. bipunctata* in practice is limited (Van Lenteren 2012; J. van Schelt, Koppert B.V., personal communication). One of the problems is that adult *A. bipunctata* tend to fly away from the crop soon after release in greenhouses (Hämäläinen 1977) and urban trees (Brakefield 1984b). Therefore, limiting its ability to disperse by flight may enhance its efficacy.

Thesis outline

In this thesis, I investigate the potential of wingless morphs of *A. bipunctata* to improve inundative aphid control by this species. It is based on multidisciplinary studies of natural variation in wing length in *A. bipunctata*. On the one hand, I examine the causes of wing reduction. On the other hand, I study the consequences of wing reduction and the potential pleiotropic effects of the wingless genes, both in an evolutionary context and in the context of biological control. The research described concerns experimental work using laboratory populations of *A. bipunctata* for which all founders originated from a Dutch population.

Part A begins to test the **efficacy of wingless *A. bipunctata* as biological control agents**

- In **chapter 2** I test the hypothesis that wingless beetles remain longer on aphid-infested plants after inundative release than winged conspecifics, and I investigate the efficacy of both morphs in aphid control. I include potential pleiotropic effects of the wingless gene on foraging behaviour.
- **Chapter 3** examines whether releases of a strain of wingless *A. bipunctata* can reduce the load of honeydew beneath urban trees produced by aphids.

Part B further **explores the variation in wing length in *A. bipunctata***. In some cases, I contrast winged to wingless morphs, whereas in others, I study in depth the variation in wing length reduction within wingless morphs. I ask how and why this variation exists, and discuss its implications for the use of *A. bipunctata* in biological control.

- I report on the relationship between the major genes regulating winglessness and melanization of wings in **chapter 4**.
- In **chapter 5**, I study how genetic and environmental factors influence the variation in wing length reduction in wingless morphs, and merge these results with previous work to provide a model for the genetic architecture of the wingless trait in the species.
- **Chapter 6** focuses on the development of the wingless character within an individual's growth from egg to adult.
- In **chapter 7**, I investigate the role of the elytra in mating behaviour, and the fitness consequences of winglessness in this context.

Part C contains a **synthesis** and an **outlook** based on the experimental work.

- **Chapter 8** summarizes and integrates the results of the previous chapters to discuss the evolution of the wingless character, and the potential of wingless *A. bipunctata* to improve aphid control. It includes suggested directions for future research.
- Finally, **chapter 9** generalizes this work by taking a perspective on utilizing intraspecific natural variation by means of selective breeding to improve natural enemies for augmentative biological control.

Acknowledgements

This introduction was improved by comments of Paul Brakefield and Peter de Jong. I am grateful to the courtesy of Hans Smid and Kjeld Olesen to use their photos. Photos in Box 3 were made with the help and equipment of Nard Dijksma (Olympus), John Lommen and Yoshi Tomoyasu (Kansas State University).

PART A

Efficacy of wingless *Adalia bipunctata* as biological control agents

2

Natural flightless morphs of the ladybird beetle *Adalia bipunctata* can improve biological control of aphids on single plants

Suzanne T.E. Lommen, Cock W. Middendorp, Carola A. Luijten, Jeroen van Schelt, Paul M. Brakefield and Peter W. de Jong

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Abstract

The challenge of using ladybird beetles for biological control of insect pests such as aphids is that the adult beetles tend to fly away from the host plants. Therefore, flightless ladybirds might improve biocontrol. There are several artificial ways to obtain flightless beetles, but it may be preferable to use natural variation in flight ability. We investigated, for the first time, biocontrol by inundative augmentation of natural flightless morphs of the ladybird beetle *Adalia bipunctata.* Microcosm experiments using single leaves with one of three species of aphid revealed no differences in consumption behaviour between flightless and winged beetles. Monitoring for 48 h of single, caged pepper plants infested with aphids of *Myzus persicae nicotianae* or *Aulacorthum solani* showed that flightless beetles had a longer residence time on the plants than winged beetles. This only translated into significantly better biocontrol of *M. persicae*. Despite their difference in residence time, both beetle morphs reduced the population growth of *A. solani*. This is probably explained by the tendency of *A. solani* to drop from the plant upon disturbance, and we predict that flightless beetles may outperform winged ones in the long term. Overall, our results provide a proof of principle that natural flightless *A. bipunctata* can improve biocontrol of aphids by ladybird beetles. However, we recognize that the effect of biocontrol will vary with the species of aphid used and that further examination in long term and large scale experiments is required.

Keywords

Adalia bipunctata, aphid pests, biological control, consumption, dispersal behaviour, escape response, flightless ladybird beetles, predator

Introduction

Predatory ladybird beetles have a long history in biological control of aphid infestations. Early augmentative biocontrol attempts made use of adult beetles that had been collected from overwintering sites or that were laboratory-reared (Majerus 1994; Obrycki and Kring 1998). However, even in greenhouses, adult beetles tend to fly away from the crop soon after release and are, therefore, not reliable biocontrol agents (Hämäläinen 1977; Minoretti and Weisser 2000). One way to increase the residence time of ladybird beetles in the crop and obtain reduction in aphid numbers is the release of non-flying juvenile stages (e.g. Ferran et al. 1996; Kehrli and Wyss 2001). However, young beetle larvae are less efficient than adults because they consume fewer aphids per day and once developed into adults they are prone to migrate if aphid densities are low (Hodek et al. 1993). Alternatively, flightless beetles can be used to prolong the residence time of adults (Ignoffo et al. 1977; Ferran et al. 1998) and might, therefore, improve biocontrol of aphids.

To obtain flightless morphs, various researchers have surgically removed wings of ladybird beetles (Ignoffo et al. 1977; Olszak 1986). Others have applied mutagenesis followed by artificial selection on wing malformations or the ability to fly in the Asian species *Harmonia axyridis* Pallas (Ferran et al. 1998; Tourniaire et al. 1999). In contrast to such artificial techniques to create flightless ladybird beetles, it could be beneficial to utilize natural variation in flight ability. Examples of species of ladybird exhibiting such variation include *Rhyzobius litura* Fabricius, where variation in flight ability results from variation in wing folding (Hammond 1985), *Adalia bipunctata* (L.), with dimorphism for wing reduction (Marples et al. 1993), and *H. axyridis*, with quantitative variation in flight ability not associated with any changes in the wings (Tourniaire et al. 2000). In the latter species, flightless strains have successfully been obtained by artificial selection for reduced flight ability (Tourniaire et al. 2000; Tomokazu Seko, personal communication).

To assess the potential of these flightless beetles to improve biocontrol of aphids, comparison to the performance of winged conspecifics is essential. So far, this comparison has only been made for selected flightless strains of *H. axyridis* by Seko et al. (personal communication). They have shown that, in small open fields, flightless *H. axyridis* have a longer residence time on the crop and are more effective in suppressing aphid numbers. So, these selected flightless beetles have potential as biocontrol agents. However, the use of predatory ladybirds is restricted to their native distribution because of the ecological risks of releasing non-native species (Koch 2003; Evans 2004; Majerus et al. 2006; Van Lenteren et al. 2008). Though *H. axyridis* has recently spread from its native area in Asia to parts of Europe and North America, where it is now abundant (Koch 2003; Brown et al. 2008), its use is at best debatable in these areas, and it is prohibited in several European countries. Therefore, natural flightless morphs of the two-spot ladybird beetle, *A. bipunctata*, are suitable candidates for biocontrol where this species is native to parts of Europe, North America,

and north and central Asia. Flightless *A. bipunctata* that lack the distal part of both pairs of wings are occasionally encountered in the wild. Since this trait is controlled genetically (Marples et al. 1993), one can easily rear pure-breeding populations from field-collected flightless individuals. In addition, the species can be reared all year round in the laboratory (Hämäläinen 1976). Hence, the use of flightless *A. bipunctata* for biological control might save production costs while it does not involve risks connected to introducing genetic modified organisms (GMOs) or non-native species.

Though Seko et al. obtained positive results for the application of flightless *H. axyridis* in biocontrol (personal communication), those results can not be directly extrapolated to flightless *A. bipunctata* because the nature of the flightlessness is different in the two species. Whereas the strain of *H. axyridis* has been selected for individuals with intact wings but reduced flight ability, natural flightless morphs of *A. bipunctata* have reduced wings which prevent flight. The genetics underlying different types of flight inability may each have different pleiotropic effects on traits that affect their suitability for biocontrol. For example, flightless *H. axyridis* that were selected for wing malformations after a chemical mutagenic treatment had 9-fold higher mortality, fecundity was reduced to a third and egg hatch rate was reduced to a quarter (Ferran et al. 1998), whereas natural flightless *H. axyridis* that were selected for normal morphology but flight inability showed no differences with wildtype beetles in these traits (Tourniaire et al. 2000). Another strain of flightless *H. axyridis* that was selected for flight inability after a chemical mutagenic treatment needed more time to search and ingest prey than wildtype beetles (Tourniaire et al. 1999). Therefore, it is necessary to specifically test the efficacy of natural flightless *A. bipunctata* in biocontrol.

Here we report on the ability of natural flightless adult *A. bipunctata* to reduce aphid pests compared to their winged conspecifics. The experiments were designed to test indoor biocontrol by inundative augmentation, where the released individuals are intended to control the pest species for a short period after release. In contrast, inoculative augmentation aims at establishing a self-sustaining population, where the progeny of the released individuals should control the pest over a longer period. However, there is little empirical and theoretical support for the success of ladybird beetles in inoculative augmentation (Obrycki and Kring 1998; Dixon 2000). From laboratory experiments it is known that longevity and life-time reproduction of flightless *A. bipunctata* are reduced to about a third of that of wildtypes (Ueno et al. 2004), but it is not known if foraging traits of flightless morphs are altered. Reduced longevity might diminish the benefits of the expected prolonged residence time of flightless beetles in inundative augmentation, whereas altered foraging traits may either enhance or hamper biocontrol. In contrast, reproduction traits do not affect the success of inundative control. We first compare the beetles' dispersal behaviour and their effect on aphid populations in a biocontrol experiment using single, caged pepper plants and two species of aphid. We then compare the food consumption of the two morphs of the ladybird beetle using three species of aphid in a microcosm experiment. Finally, we discuss the potential of flightless *A. bipunctata* for inundative biocontrol of different aphid species.

Material and methods

Ladybird beetles

Two *A. bipunctata* found in the wild in Utrecht, The Netherlands, and bearing alleles for the flightless trait, were outcrossed to over a hundred wildtypes from the same locality to obtain a laboratory population of flightless (-/- genotype for the trait) and winged ladybird beetles (+/- genotype) between 2000 and 2004. New wildtypes from the same locality were collected to establish a pure winged laboratory stock (+/+ genotype) in 2005. The populations were reared on *Ephestia kuehniella* Zeller eggs and flower pollen ad libitum and were kept at a temperature of 20.5 (±1) ºC and in a 16L:8D light regime. This temperature is higher than outdoor temperatures because the experiments focus on indoor application of ladybird beetles. Only female adult ladybird beetles were selected for use in the experiments, because they require more nutrients and spend more time on foraging than males (Hodek 1973).

Aphids

For the biocontrol experiment, we used two species that are well known pests in greenhouses and are reported to be suitable prey for *A. bipunctata*: *Aulacorthum solani* (Kaltenbach) (Mills 1981) and *Myzus persicae* (Sulzer) var. *nicotianae* (Blackman 1965). They were kept on leaves of sweet pepper (*Capsicum annuum* L. cv. Lesley) maintained on solid 1% agar solution in the bottom of Petri dishes (75 mm Ø x 30 mm) which were covered with mesh net and inverted. We used a temperature of 23.5 (±1) ºC and a 16L:8D light regime. For the consumption experiment we also used *Rhodobium porosum* (Sanderson), another pest species, which is not known as a suitable prey species. These aphids were reared in the same way but on strawberry leaves (*Fragaria x ananassa* Duchesne cv. Elsanta).

Biocontrol experiment

After eclosion from the pupae, female ladybirds were individually transferred to Petri dishes (75 mm Ø x 30 mm) containing pepper leaves, where they were allowed to feed ad lib on the target species of aphid at a temperature of 23.5 (±1) ºC. In this way we familiarized them with consuming aphids to prevent immediate dispersal when released on aphid-infested plants in the experiment. To minimize variation in the phenotype only virgins 2 to 17 days old with an approximate body length of 6 mm (corresponding to the mean size) and without any elytral tissue were used. Experiments were performed in nine cages (2.65 x 0.75 x 1.25 m) made of fine mesh net and with a solid bottom, and kept in a greenhouse with minimum temperatures of 22.5ºC and 21.5ºC in day and night, respectively. A single pepper plant (*C. annuum* cv. Lesley) with a height of 65 to 80 cm was placed on the bottom of each cage such that it was not in contact with the cage. At least six leaves of each plant were infested with in total 15 to 50 aphids of one of the species of aphid. Aphids were

allowed to settle and reproduce for one to three days and then counted. The plants were then randomly allocated to one of three treatments: flightless ladybird, winged ladybird (+/- for the trait) or control (no ladybird). In the ladybird treatments, a single beetle was placed on top of the lowest leaf of the plant. During the next 48 hours, its location (on/off the plant) was monitored at 11 time points during daytime. It was then removed from the cage and the aphids remaining on the plants counted. For *M. persicae*, each treatment was replicated 12 times, divided over six consecutive series of two replicates each. After that, 10 replicates divided over four series of two or three replicates were obtained for A*. solani*.

Consumption experiment

Microcosm experiments were performed to prevent dispersion of predators and prey, and to minimize the effect of searching ability of the predators. We used Petri dishes with sweet pepper (randomly allocated to *A. solani* and *M. persicae*) or strawberry leaves (for *R. porosum*) as described above. The experimental conditions were a temperature of 20 (±1) ºC and 16L:8D. Aphids were allowed to settle in the dishes for one day, after which their number was reduced to 10 per dish. Dishes were then randomly allocated to one of three treatments: flightless ladybird, winged ladybird (+/+ for the trait) or control (no ladybird). In the ladybird treatments, a 24-hour starved virgin female of age 8 to 12 days was introduced into the dish. Starvation was applied because the ladybirds had been reared on *Ephestia* eggs before use in the experiment and though it has been shown that they do consume aphids after switching to an aphid diet (Ferran et al. 1996), their appetite for aphids might have decreased. One day later all remaining aphids were counted. Each combination of treatment and aphid species consisted of 15 replicates.

Statistical analysis

All data were analysed using R.2.6.1. (R Development Core Team 2007). To analyze the position of the ladybird beetles in the biocontrol experiment, we fitted a GLM with ladybird morph as factor and a binomial error distribution for each time point. We then removed the ladybird morph factor from the model and compared the reduced model with the original one by a Chi-square test on the residual deviances to detect effects of ladybird morph. When a beetle could not be detected, it was regarded as a missing value for that time point.

We used an Analysis of Variance (ANOVA) on aphid population growth to analyze the effect of treatments on aphid numbers in the biocontrol experiment. Aphid population growth was calculated as final count of aphids 48 h after ladybird release over initial count of aphids at time zero. We applied an ANOVA for each species of aphid separately, since the experiments on the two species were performed consecutively. We first fitted a full factorial model with treatment and series as fixed effects and then stepwise deleted factors from this model until the minimal adequate was

found, using Chi-square tests on the residual deviances to compare models. One series consisting of two replicates was excluded from the analysis of *A. solani*, because aphid population growth in the control treatments of this series was below one, indicating unfavorable conditions.

To test the relationship between aphid population growth and ladybird residence time, we fitted a linear regression model. We used the total minimum residence time of ladybirds on the plants in daylight as deduced from cases where ladybirds were registered to be on the plant. When a data point was missing in between consistent observations on presence or absence, ladybirds were assumed to not have changed position in between, since this would require the unlikely scenario of two movements in a short period. Since ladybirds cease foraging in the dark (Hodek and Honěk 1996), residence time on the plant at night (eight hours in that time of the year) was subtracted from the total residence time. Consistent with the previously described analysis on aphid population growth, two replicates of *A. solani* were excluded.

The consumption experiment was analysed by a full factorial Generalized Linear Model (GLM) with treatment and aphid species as fixed factors. To adjust the error structure as closely as possible to the distribution of the data and to avoid overdispersion, we specified a quasi error structure with a square root link function and a variance equal to the quadratic mean. This was supported by a Box-Cox Normality plot which finds the maximum likelihood power transformation of the response variable in a regression model by the Box-Cox method. It showed that a power transformation of 0.5 (corresponding to a square root transformation) would be optimal for our data. We excluded three outliers from the analysis (one in each treatment of *A. solani*), since model checking revealed they strongly affected the results. We investigated differences between treatments by pooling two of the treatments successively and testing whether the new model was significantly different from the full model by an F test on the deviances. Because 15% of the data did not fit the assumed error structure, we re-analysed the same data with non-parametric statistics in SPSS 15.0 (Kruskal-Wallis for overall treatment effect, followed by pair-wise Mann Whitney U tests for differences between treatments where a Bonferroni correction was applied to adjust α). The results of both analyses were in agreement. To test differences in consumption of different species of aphid, the ladybird treatments were pooled and analysed separately in the same way, using y+1 as dependent variable. Similarly the control treatments were analysed separately to test differences in intrinsic population growth between the different species of aphids.

Results

Biocontrol experiment

With both species of aphid, winged beetles tended to leave the plants earlier than flightless beetles, and most of them did so within four hours after release (Fig. 1). Only two hours after release,

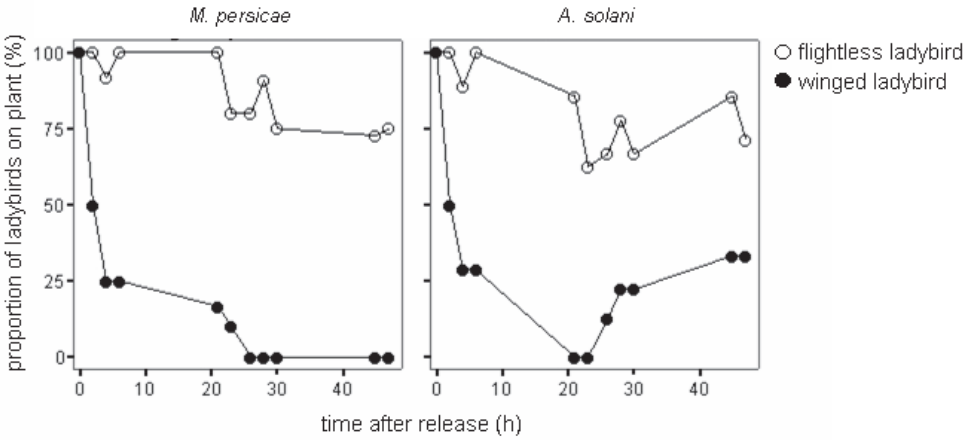


Figure 1. Location of flightless and winged beetles after release on single, caged plants infested with either *M. persicae* (left, N=8-12 plants) or *A. solani* (right, N=6-10).

flightless ladybird beetles were significantly more often found on the plant than winged beetles (*M. persicae*: p<0.01; *A. solani*: p<0.05). This distribution persisted during the remainder of the experiment, but was no longer significant after 30 h for plants with *A. solani* (p=0.053). The latter can be explained by some winged ladybirds that returned to the plants with *A. solani* (Fig. 1).

Figure 2. shows the effect of treatments on the growth of the aphid populations. There was no significant effect of series on aphid population growth in either species of aphid (*M. persicae* p=0.60; *A. solani* p=0.53), so per species all series were pooled for further analysis. The control treatments demonstrate that both species of aphid reproduced readily on the plants (mean aphid population growth >>1 in Fig. 2). Population growth was lowest when flightless ladybirds were released (mean aphid growth ± SE for flightless, winged and control treatments in *M. persicae*: 1.406 ± 0.166, 2.985 ± 0.287, and 2.433 ± 0.179, respectively; in *A. solani*: 1.339 ± 0.434, 1.526 ± 0.242, and 3.226 ± 0.362, respectively). The difference of flightless beetles with the control was highly significant for both species of aphid (both p<0.001), but flightless beetles only reduced aphid numbers significantly more than winged beetles with *M. persicae* (*M. persicae* p<0.001; *A. solani* p=0.709). This occurred because winged beetles did not produce any reduction in population growth of *M. persicae* (p=0.073), whereas they did with *A. solani* (p<0.010). Overall, only a few ladybird beetles were able to decrease the absolute number of aphids on the plants (those cases below y=1 in Fig. 3) but in no cases were all aphids removed.

The minimum residence time of the ladybird beetles on the plants in daylight was significantly negatively related to aphid population growth and explained about a third of the variation in their numbers for both species, suggesting a causal relationship (*M. persicae*: $F_{1,22}$=13.440, p<0.010, R^2=0.379; *A. solani* $F_{1,14}$=6.478, p<0.050, R^2=0.316) (Fig. 3).

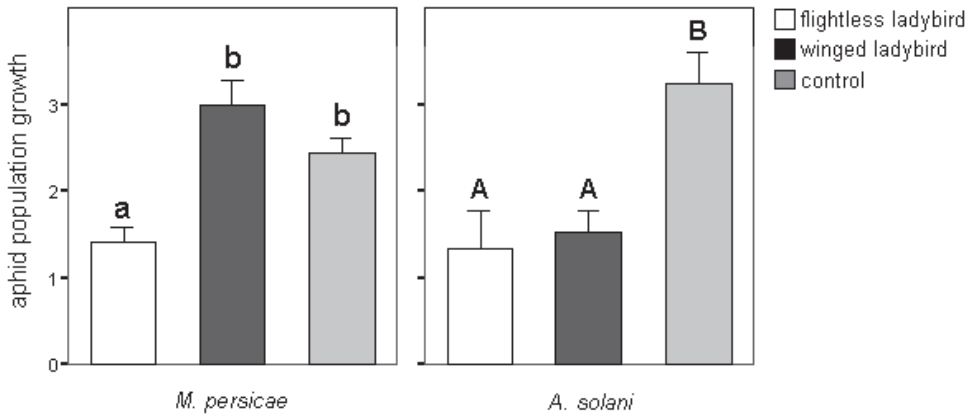


Figure 2. Aphid population growth, calculated as final/initial count, on a pepper plant infested with *M. persicae* (left panel, N=12) or *A. solani* (right panel, N=8) 48 hours after the release of flightless or winged ladybirds or the control treatment (no ladybirds). Bars represent mean values; error bars represent standard errors of the mean. Identical letters above bars indicate no significant differences between means (p>0.05).

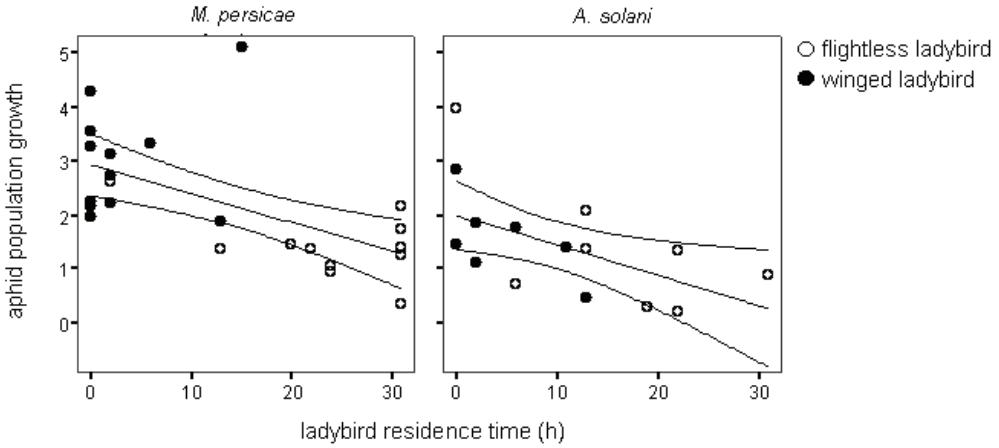


Figure 3. The relationship between aphid population growth and ladybird residence time on pepper plants infested with *M. persicae* (left panel, N=24) or *A. solani* (right panel, N=16). Symbols indicate whether flightless or winged ladybirds were released. Lines represent the overall regression lines in between the 95% confidence interval lines. Regression lines are significant (*M. persicae*: p<0.01; *A. solani*: p<0.05) and explain about a third of the variation in both species of aphid.

Consumption experiment

The consumption experiment showed a significant interaction between treatment and species of aphid (F_4=3.390, p<0.050). Nevertheless, both ladybird beetle morphs consistently reduced the number of aphids from all three species within 24 hours when offered on a leaf in a Petri dish (Fig. 4) (F_3>7.190 for each ladybird morph, p<0.001). There was no significant difference between the numbers of aphids eaten by winged and flightless beetles (F_3=0.516, p=0.672).

Comparing the consumption of the different aphid species, there was no difference between *M. persicae* and *A. solani* (median of remaining aphids of *M. persicae* = 0; median *A. solani* = 0.5; F_1=0.025, p=0.875) but the ladybird beetles ate higher numbers of those species than of *R. porosum* (median *R. porosum* = 8; $F_{1=}$44.271, p<0.001). The control treatments show that populations of *M. persicae* and *A. solani* grew at the same pace (median aphid number *M. persicae* and *A. solani* = 12; F_1=0.050, p=0.825), but faster than those of *R. porosum* (median *R. porosum* = 10; $F_{1=}$8.408, p<0.010).

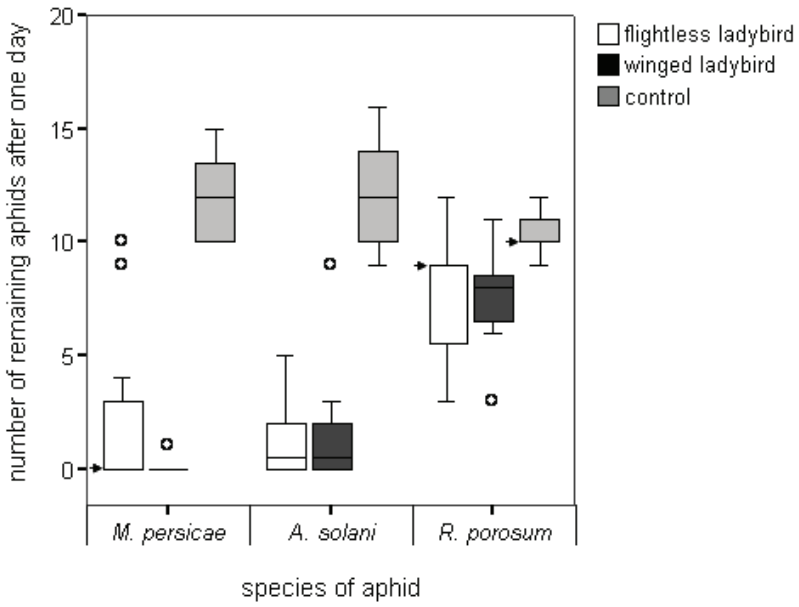


Figure 4. Number of remaining aphids in a Petri dish one day after a flightless ladybird beetle, a winged ladybird or no beetles (control) were introduced in a dish with 10 aphids of one of three species (N=15 for *M. persicae* and *R. porosum*, N=14 for A. *solani*). Boxes represent interquartile ranges (IQR), covering 50% of all data points, lines in boxes or arrows indicate median values, whiskers show the range of values that fall within 1.5 IQR of the box, and circles represent data that fall outside this range. There were no significant differences between the two ladybird treatments (p>0.05), and both ladybird treatments significantly reduced the number of aphids compared to the controls (p<0.001). Fewer *R. porosum* were consumed than *A. solani* or *M. persicae* (p<0.001), and *R. porosum* grew slower than both of the two other species of aphid (p<0.01).

Discussion

Proof of principle

The results of the biocontrol experiment show that flightless beetles stay longer on single, aphid-infested plants than winged beetles regardless of the species of aphid. They consistently reduced aphid population growth compared to the controls for both species, and were more successful in control of *M. persicae* than winged beetles, because winged beetles did not have any effect on population growth of this species. The effective control of *M. persicae* can be explained by the longer residence time of the flightless beetles on the plants, which is inversely related to aphid growth.

In contrast, flightless beetles were not significantly more successful than winged beetles in controlling *A. solani* because both ladybird morphs reduced population growth of this species. This can probably be explained by a tendency of *A. solani* to drop from the plant upon disturbance as defense behaviour, in contrast to *M. persicae* that does not tend to drop (unpublished results). This dropping behaviour is a well known escape response of aphids that can be elicited by the presence of a predator or parasitoid. Ladybird beetles are among the species of predator that cause highest displacement by dropping (Losey and Denno 1998; Day et al. 2006). This behaviour increases mortality risk of the aphid from other sources (Dill et al. 1990) and has been shown to contribute to the reduction of aphid population growth in field experiments (Nelson et al. 2004). As ladybird beetles show negative geotropism and climb to the top of the plant to take off for flight (Bänsch 1966; Hodek 1973), the winged ladybird beetles in our experiment are likely to have induced dropping behaviour before they left the plants. In addition, in our cages some winged ladybirds returned to the plants after they had left them, whereas this is unlikely in an agricultural cropping system. Therefore, the experimental results for *A. solani* might be conservative. Residence time of the ladybirds was also significantly inversely related to aphid growth of *A. solani*, suggesting that a longer residence time does contribute to aphid removal. Due to the short duration of the experiment, this contribution was probably small compared to the effect of the presence of ladybirds after release. However, in the long term, flightless beetles may well outperform winged beetles regardless of the aphids' tendency to dropping behaviour, because their longer residence time is likely to increase aphid displacement by both consumption and dropping behaviour.

Together, these results form the proof of principle that flightless *A. bipunctata* indeed stay longer on the plants and, as a consequence, might improve biocontrol by reducing aphid population growth.

This result was also found for flightless *H. axyridis* released on experimental outdoor fields of eggplants infested with the cotton aphid, *Aphis gossypii* (Tomokazu Seko, personal communication). These ladybirds had been artificially selected for reduced flight ability without wing malformations, in contrast to the flightless *A. bipunctata* which are flightless as a result of wing reduction. Though the cause of flightlessness is different for the two species, the results with respect to residence time and biocontrol is similar. Apparently, possible negative pleiotropic effects of the genes causing flightlessness are outweighed by the positive effects of longer residence time. The similarity in the results of two different species of ladybirds, together with the relationship between residence time and reduction of aphids demonstrated in this paper, strengthens the idea that flightless beetles can improve the efficacy of ladybirds in control of aphids.

Properties of predator and prey species affect success of biocontrol

Besides predator dispersal behaviour, the predator's ability to remove prey from the host plants is another important factor determining the effectiveness of biocontrol. Many properties of both predator and prey affect this ability, such as searching efficiency and consumption behaviour of the ladybirds, and growth rate and defense mechanisms (including the previously discussed escape behaviour) of the aphids. Therefore, we compared the consumption behaviour of flightless and winged ladybird beetles using one of three species of aphid in microcosms. The control treatments without beetles allowed us to examine aphid population growth rates.

The results of the consumption experiment show no significant differences in feeding behaviour between flightless and winged beetles (homozygote recessive (-/-) and dominant (+/+) for the trait, respectively). Therefore, we also expect winged beetles that are heterozygote (+/-) for the trait, as used in the biocontrol experiment, to behave similarly. Consequently, we assume that consumption behaviour did not contribute to the differences between flightless and winged beetles treatments observed in the biocontrol experiment. However, experiments with larger numbers of aphids may reveal differences in consumption and we cannot exclude differences in other aspects of foraging behaviour, such as searching efficiency. For example, Tourniaire et al. (1999) showed that their strain of artificial flightless *Harmonia* was slower in encountering and ingesting aphids on plants than wildtype conspecifics.

In contrast, we have observed differences between the prey species. First, though *A. bipunctata* is known to accept a wide range of prey species (Hodek 1973), the number of aphids consumed by the ladybirds when offered in a Petri dish showed variation: *M. persicae* and *A. solani* were consumed at substantially higher rates than *R. porosum*. By restricting space and time, we minimized the effect of searching ability of the ladybird beetles, and of population growth and defense behaviour of the aphids. Therefore, observed differences are likely to represent the variation in the ladybirds' readiness to eat various species of aphid. Consequently, we predict the effectiveness of flightless

A. bipunctata in controlling *R. porosum* to be minimal. However, we can not rule out potential effects of the host plant, which was different for *R. porosum*. As flightless beetles behaved similarly to wildtypes in their consumption behaviour, existing literature on feeding habits of wildtype *A. bipunctata* can be consulted to predict the success of biocontrol of a particular species of aphid by flightless morphs. Secondly, the species of aphids varied in population growth rate, which becomes relevant to biocontrol if flightless ladybirds do not remove all aphids from the plants immediately (Minoretti and Weisser, 2000). Together, these results highlight the potential for wide variation in the effectiveness of biocontrol of different species of aphid by flightless ladybird beetles.

Optimizing flightless beetles for use in biocontrol

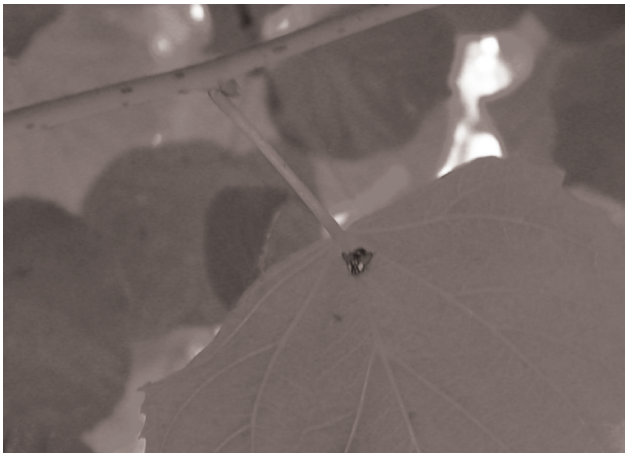
Although flightless ladybird beetles reduced aphid population growth significantly in the biocontrol experiment, the effectiveness of biocontrol may be optimized through further investigation. For example, none of the beetles was able to eradicate the aphid population on the plant. These results are in line with Minoretti and Weisser (2000), who showed that individual *Coccinella septempunctata* beetles did not remove all individuals of small pea aphid colonies (10 to 30 aphids) from bean plants before they left the plants (which was within two hours after release). In a long term study with low densities of the cotton aphid on outdoor eggplants, flightless *H. axyridis* were able to keep aphids at the initial densities, but also never eradicated aphid populations (Tomokazu Seko, personal communication). In contrast, Olszak (1986) reported that two manually de-winged adults of *A. bipunctata* succeeded in removing small colonies (10 or 20 aphids) of *Aphis pomi* from single, caged apple trees in seven days, but the predators were replenished when they escaped. Theoretical work predicts that ladybird females will not consume aphid colonies completely at the place where they oviposit, in order to guarantee food availability for their offspring (Dixon 2000). We should monitor over longer periods to examine whether absolute aphid numbers would decrease when flightless ladybirds are given more time. In addition, it is known that the predation pattern of ladybird beetles is scale-dependent (Ives et al. 1993) and, therefore, experiments should be conducted at larger scales resembling agricultural settings, such as greenhouses. Further, we would have to apply various predator densities to determine the optimum in aphid reduction. Since there was substantial variation among individual ladybird beetles, it might be better to introduce more than one beetle at every release spot. Finally, the flightless phenotype may be improved by artificial selection on traits important for biocontrol. In addition, even though flightless *A. bipunctata* represent a natural phenotype, we should also investigate how large scale releases of this morph would alter allele frequencies of natural *A. bipunctata* populations.

Conclusion

Our experiments have demonstrated the proof of principle that naturally flightless morphs of *A. bipunctata* remain longer on aphid infested plants and, as a consequence, can reduce aphid numbers more than their winged conspecifics. This longer residence time does not translate into more effective biocontrol in the short term when the particular species of aphid involved has a high tendency for dropping behaviour, but we predict positive results in the long term. In line with previous experiments and theory, the flightless beetles were not able to eradicate the aphids. Nevertheless, they kept aphid numbers close to their initial densities. We have shown that flightless ladybirds were not different from wildtypes with respect to their feeding behaviour, suggesting that knowledge on the prey range of wildtypes can be applied to the flightless morphs. In addition, we have pointed out that there are many differences between aphid species that can affect the success of biocontrol, that is, their defense mechanisms, their population growth rates, and the readiness of the ladybirds to eat them. Hence, whilst flightless ladybirds may improve biocontrol, further research is needed to examine whether the reported effects will prevail in larger, realistic agricultural settings and for longer periods with different species of aphid.

Acknowledgements

We thank Chantal Bloemhard for samples of *R. porosum*, Harald van Mil for advice on the statistical analysis and three anonymous reviewers for their constructive comments.



The lime tree experiment in Amsterdam

3

Releases of a natural flightless strain of the ladybird beetle *Adalia bipunctata* reduce aphid-born honeydew beneath urban lime trees

Suzanne T.E. Lommen, Thomas C. Holness, Alfons J. van Kuik, Peter W. de Jong, Paul M. Brakefield

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Abstract

Aphids can cause major environmental problems in urban areas. One important problem is the annual outbreaks of lime aphid, *Eucallipterus tiliae* (L.) (Hemiptera: Aphididae), which spoil the surroundings of lime trees by depositing honeydew. To date no environmentally friendly method has been demonstrated to yield effective control of lime aphids. Attempts are made in some cities to control lime aphids by releasing larvae of the native two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae). However, it is known that adult ladybird beetles disperse soon after release, and there is little indication they provide control of the aphids. Here, we demonstrate experimentally that releases of a flightless strain of *A. bipunctata,* obtained from natural variation in wing length, can reduce the impact of honeydew from lime aphid outbreaks on two species of lime in an urban environment. Both larvae and adult beetles were released, and we discuss the contribution of the flightless adults to the decline in honeydew.

Keywords

Coccinellidae, biological control, dispersal, Eucallipterus tiliae, inundative control, wing polymorphism

Introduction

Aphids are well known as pest insects in agricultural and ornamental crops (Minks and Harrewijn 1987), but they can also have a major environmental impact in urban environments (Dixon 1971a, b; Dreistadt and Dahlsten 1988). One such problem is the pollution caused by honeydew deposits from aphid outbreaks on urban trees (Dahlsten et al. 1999). Aphids secrete surplus plant sap taken up from their host plant as honeydew, a sticky solution rich in sugars. When aphid numbers in urban trees are high, the excreted honeydew drips down to spoil the environment below and around the trees (Mawson 1948). Not only do benches and parked vehicles become sticky, but pavements also become dangerously slippery in damp weather conditions, posing a risk especially for the elderly. One important species causing such a nuisance is the monophagous aphid, *Eucallipterus tiliae* (L.) (Hemiptera: Aphididae), on lime trees (*Tilia* spp.) (Malvales: Malvaceae), which are historically popular plants in urban areas. *Eucallipterus tiliae* is abundant throughout the Palaearctic region and has been introduced to North America and New Zealand (Blackman and Eastop 1994; Zuparko and Dahlsten 1996). Aphid outbreaks normally occur in each year and populations typically peak once per year in cool climate zones, and up to two times in warmer climate zones. However, the timing, intensity, and duration of these peaks vary dramatically (Dixon 1971c; Dahlsten et al. 1999). Field observations and theoretical modelling indicate that naturally occurring predators and parasitoids may affect population growth of the lime aphids, but fail to control it (Wratten 1973; Dixon and Barlow 1979; Glen and Barlow 1980; Zuparko and Dahlsten 1995; Dahlsten et al. 1999; Dixon 2000). As a result, outbreaks of lime aphids frequently cause severe problems of honeydew deposits in urban areas. The large sums of money spent by numerous local authorities in The Netherlands to reduce this excess honeydew illustrate the substantial environmental impact (H. Kuppen and M. Visser, personal communication).

The need for biological control of insect pests in urban areas is evident from an environmental and health perspective. To date, the only effort to obtain biological control of lime aphids that has been reported, is the release of parasitoids in North America. None of those species has been shown to regulate the aphid populations and reduce honeydew satisfactorily (Zuparko and Dahlsten 1995, 1996; Dahlsten et al. 1999). In Europe, the predatory two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) is most commonly employed, but the effect of its release in lime trees has never been documented in scientific literature. The few examples of successful augmentative control of aphids by ladybirds in other systems in the open air (reviewed by Powell and Pell 2007; Obrycki et al. 2009), included one where released *A. bipunctata* controlled populations of the aphid *Dysaphis plantaginea* (Passerini) (Hemiptera: Aphididae) in apple trees (Wyss et al. 1999a; Kehrli and Wyss 2001). The flight dispersal of the adult ladybirds away from the target plants is thought to be a major obstacle (Kieckhefer and Olson 1974; Ignoffo et al. 1977; Obrycki and Kring 1998) with

capture-recapture experiments on *A. bipunctata* indicating that the majority of the adults released in lime trees emigrate within a few days (Brakefield 1984b).

Limiting dispersal and thus prolonging the residence time of the ladybirds released could contribute to a more effective reduction of aphid population growth. One way to obtain a prolonged presence of ladybirds is the release of the flightless, larval stages rather than the adult stage. This is in fact common practice for *A. bipunctata* in honeydew management (M. Visser, personal communication). Practitioners, however, report that the release of these larvae does not result in a higher number of adults later in the season, compared to trees that were not provided with larvae (H. Kuppen, personal communication). This could be explained by dispersal of the adult beetles as observed for the Asian ladybird beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) by Trouvé et al. (1997).

Another way to limit adult dispersal by flight is to use flightless adults. (Ignoffo et al. 1977) have proved this concept by showing that de-winged adults of *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) remained significantly longer in a soybean crop and consumed more prey than untreated, winged beetles. In the past fifteen years, several flightless populations of *H. axyridis* and *A. bipunctata* have been developed in different ways (Ferran et al. 1998; Tourniaire et al. 1999; Ueno et al. 2004; Seko et al. 2008; Ohde et al. 2009). When released in crops in protected and open cultures, they have shown longer residence times, and often better control of aphids compared to their conspecifics capable of flight (Ferran et al. 1998; Lommen et al. 2008; Seko et al. 2008).

In this paper, for the first time we report the biological control of aphids outdoors by a flightless strain of *A. bipunctata*. *Adalia bipunctata* has a high preference for the lime aphid (Kalushkov 1998) and is a major natural enemy of this species. In contrast to the ladybird beetle *H. axyridis*, *A. bipunctata* is native to large parts of Europe and North America. Wingless adult morphs of *A. bipunctata* occur naturally, but rarely (Majerus and Kearns 1989; Marples et al. 1993). Both pairs of their wings are truncated to a variable extent (Lommen et al. 2009). Since winglessness in *A. bipunctata* is genetically controlled by a recessive allele at a single major locus (Marples et al. 1993), it is straightforward to rear pure-breeding stocks from wingless specimens collected in the field. Early experiments on single, aphid-infested, pepper plants in the greenhouse showed that wingless morphs have a longer residence period on the plant. As a consequence, they can improve biological control of aphids compared to their winged, wildtype conspecifics in an enclosed environment (Lommen et al. 2008). In this paper, we examine experimentally how releasing individuals of this wingless strain of *A. bipunctata* on two species of lime tree in a Dutch city affects honeydew deposits from lime aphid outbreaks.

Material and methods

Ladybirds

Wingless *A. bipunctata* originated from a laboratory stock established between 2000 and 2004 by outcrossing two individual ladybird beetles bearing alleles for the wingless trait to wildtypes from the same location near Utrecht in The Netherlands (details in Lommen et al. 2005). They were bred on a diet of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs and flower pollen (De Clercq et al. 2005) at a temperature of 20.5 (±1) °C and in a 16L:8D light regime.

Location

The experiment was performed in the Slotervaart district in the city of Amsterdam, The Netherlands, where lime trees are the dominant tree species and where severe problems with honeydew are experienced each year. We chose two areas, one km apart, one planted with the common lime, *Tilia* x *vulgaris* Hayne (synonym: *T.* x *europaea* L., hybrid of *T. cordata* and *T. platyphyllos*) and another with the small-leaved lime, *T. cordata* Mill. These species were found to be most susceptible to the lime aphid (Carter and Nichols 1986; Zuparko and Dahlsten 1994).

Experimental design

Figure 1 gives a schematic overview of the spatial set-up of the experiment. In each area, we selected free-standing trees hosting lime aphids in low numbers but with very few *H. axyridis* and without ants in late May 2008 by eye. We assigned pairs of trees, separated by less than 10 m and similar in size and appearance, to replicate blocks. In this way we created five pairs of *T.* x *vulgaris* distributed over three streets, and three pairs of *T. cordata* distributed over two streets. Stem circumferences of all 16 trees were measured at breast-height. One tree of each pair was randomly chosen to receive the ladybird treatment and the other served as a control.

Table 1 gives an overview of the timing of honeydew monitoring and the application of ladybird treatments. The experiment started on May 29th (day 0) by measuring the honeydew and initiating the ladybird treatment. One pair of *T.* x *vulgaris* trees had to be replaced because we found ants attacking the ladybirds on release, and one pair of *T. cordata* trees could not be treated and measured that day because of rainfall. Hence, a new pair of *T.* x *vulgaris* and the untreated pair of *T. cordata* received the first ladybird treatment and were measured for the first time only four days later (Table 1).

The ladybird treatment involved the release of 150 wingless *A. bipunctata* per tree by attaching three open tubes with beetles to the stem of the tree up to one meter below the lowest branches. Subsequent ladybird treatments were applied on the same trees on seven other occasions at intervals of about 1 week over the next two months. Initially, insufficient adults were available,

and therefore, only larvae (third and last instar) were released. In subsequent treatment occasions, mainly adults or only adults were released (Table 1).

Measures of honeydew deposits were made during the first two months and once after three months (Table 1). On this final occasion one pair of trees had been trimmed and was therefore not measured. Honeydew falling from the trees was measured by placing eight pieces of water-sensitive paper (76 x 26 mm) around the stem 60 cm away from it (for two pairs of small trees 40 cm was used), each in one of eight wind directions. Honeydew drops produce blue dots upon contact with the paper. After one hour of exposure, the papers were removed and water-free stored, and the blue dots were then counted in the laboratory. To determine the repeatability of counting dots, the observer once recounted drops on all eight papers of eight different trees collected on one of the dates.

Data about weather conditions were obtained from the nearest weather station from the Royal Netherlands Meteorological Institute (http://www.KNMI.nl).

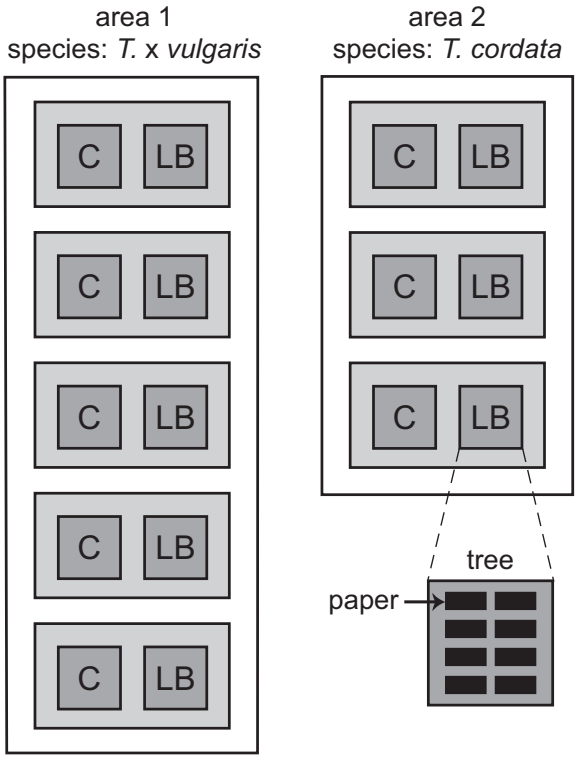

Figure 1. Schematic overview of the spatial set-up of the experiment. White blocks represent areas, light grey blocks within areas are paired trees, dark grey squares are individual trees receiving a ladybird treatment (LB) or serving as controls (C), and black rectangles within trees are paper samples.

Table 1. Overview of the timing of the experiment. Day 0 corresponds to May 29th 2008, and crosses in the second and third column indicate when honeydew was monitored and ladybird treatments were applied, respectively. The last three columns give the number of ladybirds per developmental stage released.

day	monitoring	treatment	numbers of ladybirds released		
			larvae	adults	total
0 [(a)]	x	x	150	0	150
7	x	x	56	94	150
14		x	56	94	150
18	x				
22	x	x	56	94	150
28	x	x	0	150	150
35	x	x	0	150	150
42	x	x	0	150	150
54	x	x	0	150	150
60	x				
91 [(b)]	x				

a) Two pairs of trees (one of each species) were monitored and treated at day 4 instead
b) One pair (of *T.* x *vulgaris*) was not monitored

Statistical analysis

All statistical analyses were conducted using R software version 2.8.1. (R Development Core Team 2007). We determined the repeatability of honeydew counts for each paper and for the total of eight papers per tree. To analyse honeydew levels, the first measurement of each pair of trees was considered the zero measure (taken on day 0 for six of the pairs, and on day 4 for the remaining two pairs). We first tested whether the initial honeydew level depended on the size of the tree by regressing the zero-measurement on tree stem circumference. In this analysis, we used the sum of the eight honeydew counts per tree as measure of the honeydew level and we pooled data from both species of tree, because of the limited sample sizes and the similar pattern for the two species. We then analysed honeydew deposits over time by analysing data of each monitor day separately. We fitted Generalised Linear Mixed Effect Models (GLMMs) using a Poisson distribution to deal with the count data (R package: lme4, function: lmer). We defined as fixed factors the explanatory variables: tree species and treatment, and their interaction term. We specified the other explanatory variables as random effects hierarchically nested according to their spatial scale: papers within tree, trees within pair, and pairs within tree species. Since stem circumference was not related to honeydew level, this factor was not included in the analysis. We stepwise deleted non-significant fixed factors from the full model while the random factors were kept the same, until the minimal adequate GLMM was found.

The factor significance levels were obtained from a Chi-square test on the deviances of the models, as appropriate for count data. They were compared to the alpha values that were adjusted following Holm-Bonferroni to correct for the multiple measurements taken on the same objects.

Results

Repeatability of the honeydew counts was 0.979 for the individual papers and 0.993 for the total drops of the eight papers per tree. Trees had a stem circumference of 67 ± 5 cm (mean ± SE) and variation in size was not correlated with initial honeydew levels (ANOVA, $F_{1,14} = 1.775$, p=0.204). Therefore, tree size was not included as co-factor in the analysis of honeydew deposits over time.
Figure 2 displays honeydew levels over time. In general, the pattern was extremely similar for both species of tree. Honeydew levels of control trees increased from the start of the experiment until a maximum at day 18. After that, levels declined until those of the zero-measure were approached, 60 days after the start of the experiment. In contrast, treated trees showed a steady decline soon after the introduction of the ladybirds until the initial honeydew level was reduced to 32% on day 60. At that time, honeydew amounts were half of those beneath the control trees. A month later, honeydew levels of the treated trees had on average increased slightly, but remained about 60% of that observed for the controls (Fig. 2a). The slope of the decline in honeydew from day 18 to day 60 deposits is similar for control and treated trees (Fig. 2a).
Table 2 summarizes the results of the corresponding GLMM. There was no significant effect of treatment, tree species, or their interaction term on the initial honeydew level of the trees (zero-measurements). After the ladybird treatment was initiated, no significant interaction between treatment and tree species was found on any of the following monitoring days. Although *T. cordata* had slightly lower values of honeydew than *T.* x *vulgaris* throughout the first two months of the experiment, and slightly higher values three months after the start of the experiment, this difference was never significant. From day 7 on, honeydew deposits from ladybird-treated trees were lower than for the control treatment (Fig. 2a), and this difference was significant from day 18 to 28 and on day 60 and 91 (Table 2). In the other periods, the same trend was observed and was nearly significant (Table 2). This effect can also be seen within all but one of the pairs separately (Fig. 2b-i). One atypical pair showed higher levels of honeydew in the treated than in the control tree throughout the entire experiment (Fig. 2f). However, this ladybird treated tree began with three times the amount of honeydew compared to that of its paired control, and as in the other treated trees, the honeydew level dropped soon after the start of the experiment and had decreased to 59% after 2 months. Thus, although it never dropped below that of the paired control, the change in honeydew fits the general pattern. One other ladybird treated tree shows a deviating pattern: it began with a similar extreme

high amount of honeydew, but it achieved lower honeydew values than its control after three weeks, which then further decreased dramatically to only 14% after two months (Fig. 2c).

The weather was very variable that summer, and occasionally it rained heavily (average day temperature was 12.4-24.2°C; rainfall was 70% higher in July and August than normal).

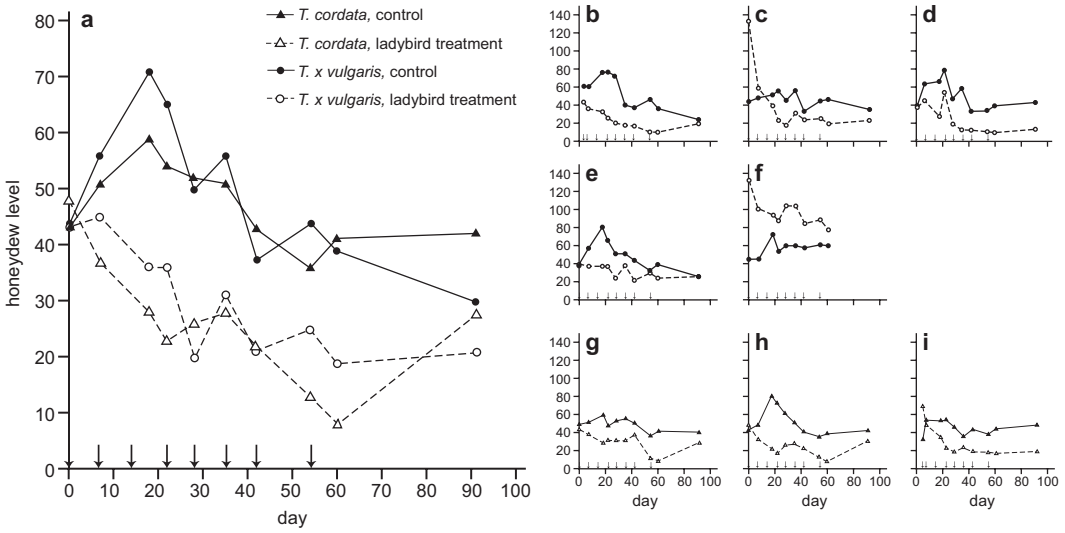

Figure 2. Honeydew level over time for ladybird treated (open symbols and interrupted lines) and control trees (filled symbols and lines) for trees of *T. cordata* (triangles) and *T. x vulgaris* (circles). Arrows indicate when ladybird treatments were applied. Panel a. Symbols represent median values of the total number of honeydew drops per tree for each combination of tree species and treatment. Panel b-i. Each panel corresponds to one pair of trees of *T. x vulgaris* (b-f) or *T. cordata* (g-i) and symbols represent the total of all honeydew drops per tree.

Table 2. Effect of treatment and tree species on honeydew beneath aphid-infested lime trees. Results of the GLMM are listed for the initial measurements and each of the following monitor days separately. Significant p-values are displayed in bold. Alpha-values were adjusted following Holm-Bonferroni.

day	explanatory variables											
	treatment x tree species				tree species				treatment			
	X^2	df	p	α	X^2	df	p	α	X^2	df	p	α
0[(a)]	0.183	1	0.699	0.013	0.641	1	0.423	0.013	2.816	1	0.093	0.025
7	0.800	1	0.371	0.008	1.693	1	0.193	0.007	0.580	1	0.446	0.050
18	1.168	1	0.280	0.006	2.050	1	0.152	0.006	10.032	1	**0.002**	0.006
22	1.573	1	0.210	0.006	3.124	1	0.077	0.006	8.480	1	**0.004**	0.007
28	0.164	1	0.685	0.017	0.175	1	0.675	0.025	7.458	1	**0.006**	0.008
35	0.091	1	0.763	0.050	0.494	1	0.482	0.017	4.736	1	0.030	0.017
42	0.367	1	0.545	0.010	0.013	1	0.910	0.050	6.001	1	0.014	0.013
54	1.131	1	0.288	0.007	1.385	1	0.239	0.008	6.246	1	0.012	0.010
60	2.363	1	0.124	0.005	1.137	1	0.286	0.010	10.083	1	**0.001**	0.006
91	0.133	1	0.715	0.025	4.360	1	0.037	0.005	11.173	1	**0.001**	0.005

a) For two of the pairs, measures were taken at day 4 instead

Discussion

Factors affecting honeydew deposits

Our results provide proof of principle that releasing individuals of a strain of wingless *A. bipunctata* in urban lime trees can effectively reduce honeydew deposits beneath these trees. This effect was observed as early as one week after introduction of the ladybirds, but became significant only after 18 days in mid June (Table 2). Honeydew levels then remained lower in treated trees during the two months in which ladybirds were released (Fig. 2a). In late August, one month after the final release, the treatment effect remained significant.

Although Heimbach (1982) observed that the daily honeydew production of a lime aphid is highest in midsummer and lower in spring and late summer, honeydew levels are likely to reflect aphid densities in the tree (Dreistadt and Dahlsten 1988). Thus, we can infer that aphid populations in the control treatment expanded rapidly after the start of the experiment late May, but ceased three weeks later when honeydew levels started to decline. Such a pattern corresponds to the known cycle of *E. tiliae* (Dixon 1971c). In contrast, the treated trees showed a steady decline in honeydew in the same period. Therefore, we conclude that the release of ladybirds has hampered the growth of the aphid populations.

How the ladybirds caused a reduction in honeydew compared to the control treatment might be explained by several mechanisms that are not mutually exclusive: by consumption of aphids, by displacement of the aphids, or by the induction of dispersive aphid morphs (Wratten 1976; Losey and Denno 1998; Dixon and Agarwala 1999; Weisser et al. 1999; Day et al. 2006).

After the first three weeks, the pattern was similar in control and treated trees: we observed a decline in honeydew during the next six weeks over which ladybirds were released, followed by little change over the final month (Fig. 2a). Therefore, we cannot confirm whether the ladybird treatment contributed to the continuation of the honeydew decline after day 18. However, it is unlikely that aphid populations in the treated trees further declined naturally after day 18. Such crashes normally only occur when host plants are overpopulated (Dixon 2000), whereas the size of aphid populations in the treated trees was already halved after three weeks, when the decrease continued. Thus, it is likely that the aphid population in the treated trees kept declining after day 18 because of the ladybirds released.

Unfortunately, we cannot disentangle the contribution to honeydew decline by the adults of the flightless ladybird strain (released at day 7-54) from that of the larvae (released at day 0-22). Therefore, we cannot confirm the contribution of the adult stages of flightless *A. bipunctata* to the honeydew decline. It thus remains unclear whether the observed effects can be attributed to the flightless characteristic of this strain.

Finally, other biotic and biotic factors may have affected the amounts of honeydew. Local insect

communities in the trees could interact with the aphids and the ladybirds released. The heavy showers might have washed off the honeydew from the leaves, and washed off or disturbed the aphid populations (Mann et al. 1995), resulting in lower honeydew levels in the following days. This may explain part of the observed decline in honeydew. Fluctuations in the second month might be attributable to differential weather conditions between measurements.

Altogether, the experiments should be repeated and expanded to investigate the contribution of adults of the flightless strain to honeydew decline. Sampling *E. tiliae*, *A. bipunctata,* and the rest of the insect communities in, and around the trees, in such research would give more insight in the underlying ecological mechanisms. However, it is impossible to get accurate quantifications without disturbance.

Management of honeydew by *Adalia*

Our research provides a proof of principle for augmentative control of lime aphids by a strain of flightless *A. bipunctata*. It is important to realize that we aimed for inundative control (where many released individuals are expected to control the pest) rather than inoculative control (where a few individuals are released and the progeny is expected to control the pest). Inoculative control with ladybirds is generally difficult, because ladybirds have a much longer generation time than their aphid prey (Dixon 2000). Also, we do not expect that release of flightless ladybirds in one year will have an effect on the aphid population the following year, because the year-to-year dynamics of aphid populations in lime trees are mainly regulated by intraspecific factors (Dixon 2000).

Further research is needed to examine whether the application of flightless *A. bipunctata* in lime trees can be refined with respect to 1) the frequency of application, 2) the timing of application, and 3) the numbers and stage of ladybirds released. The release frequency used in our experiment is too high to be cost-effective in tree-aphid management. Especially the high frequency of releases involves high costs. Therefore, it is necessary to determine whether the same reduction in honeydew can be achieved by fewer releases of ladybirds. Because ladybird treatments were applied on a weekly basis in each of the treated trees, we cannot evaluate the added contribution of each release and cannot draw conclusions about the best timing of ladybird release. Repeated releases might not be required to obtain similar results. The timing of the release is crucial for successful control. Many studies show that augmentative releases of ladybirds should be carried out early in the year before aphid populations build up (Ferran et al. 1996; Trouvé et al. 1997; Wyss et al. 1999a). The numbers of ladybirds released might be adjusted to the release frequency and the tree size. It is more effective to release larvae rather than adult ladybirds with respect to the costs of mass rearing. The release of larvae may also prolong the presence of ladybirds in the trees, although Adachi-Hagimori et al. (2011) found a higher dispersal rate of larvae compared to adults of a flightless strain of *H. axyridis*, after release in aphid-infested *Brassica* in the greenhouse.

Ladybirds are unlikely to reduce honeydew levels completely. Therefore, social studies should be made to confirm whether the effect of flightless *A. bipunctata* is sufficient to reduce complaints from urban residents.

Flightless *Adalia* compared to wildtypes

Commercial companies will call for evidence that wingless morphs of *A. bipunctata* are more efficient in biological control of lime aphids than the winged ones that are already commercially available, before they switch to the use of the wingless morph. However, comparative research is difficult in high trees and in open areas where natural populations cannot be excluded. Another study with the same populations of *A. bipunctata* in the greenhouse showed that the wingless morphs were better in controlling an aphid species on pepper plants (Lommen et al. 2008). Similarly, flightless morphs of *H. axyridis* also performed better in this respect on vegetables outdoors than those capable of flight (Seko et al. 2008).

Commercial companies have also raised concerns regarding the fitness of wingless *A. bipunctata*, which is lower than that of wildtypes (Ueno et al. 2004), and the cost involved in mass rearing this wingless morph. There is scope for improvement of the fitness of wingless *A. bipunctata.* The laboratory population of wingless *A. bipunctata* displays wide, heritable variation in the degree of wing reduction (this thesis, chapter 5). This variation is correlated to variation in fitness traits, in such a way that individuals with the mildest reductions in wings (but still not able to fly) and elytra have higher fitness than those with strong reductions (Ueno et al. 2004). We have successfully obtained populations with mild wing reduction with a few generations of artificial selection (this thesis, chapter 5). Such populations are expected to have a higher fitness than the unselected stock population, and this should ease mass rearing.

Concluding remarks

Our results are promising for the management of aphids in urban environments with this strain of flightless *A. bipunctata*. Interestingly, urban residents in California were willing to pay substantially more to manage insects in nearby trees by biological control than by chemical pesticides or biological insecticides (Jetter and Paine 2004). It may also be advantageous that the flightless character of this strain of *A. bipunctata* originates from a natural population. Finally, using flightless morphs of *A. bipunctata* has an advantage compared to those of *H. axyridis*, because the latter is an introduced, exotic species in many of the areas where lime trees are planted, and the putative environmental risks involved are still being debated (Van Lenteren et al. 2008; Kindlmann et al. 2011). Generally, our research adds to the growing evidence that flightless ladybirds can be effective in augmentative control of different aphid species on several crops in a variety of environments (Kuroda and Miura 2003; Lommen et al. 2008; Seko et al. 2008; Iguchi et al. 2012), including an outdoor, urban environment.

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PART B

Causes and consequences of variation in wing length in *Adalia bipunctata*

A melanic, wingless male mating a typical, winged female

4

Genetic linkage between melanism and winglessness in the ladybird beetle *Adalia bipunctata*

Suzanne T.E. Lommen, Peter W. de Jong, Kees G. Koops, and Paul M. Brakefield

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Abstract

We report a case of genetic linkage between the two major loci underlying different wing traits in the two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae): melanism and winglessness. The loci are estimated to be 38.8 cM apart on one of the nine autosomes. This linkage is likely to facilitate the unravelling of the genetics of these traits. These traits are of interest in the context of the evolution of intraspecific morphological diversity, and for the application of ladybird beetles in biological control programs.

Keywords

Adalia bipunctata, classical genetics, linkage disequilibrium, wing morphology

Introduction

Variation in insect wing morphology has long been studied from different perspectives. Examples include both fundamental studies in the context of the evolution and development of phenotypic variation, and applied studies in the context of pest control. The genetics underlying such variation can help to better understand the nature of the variation in either of the contexts. We report here genetic linkage of two morphological wing traits, melanism and winglessness, in the ladybird beetle, *Adalia bipunctata* (L.).

Melanism in ladybirds

Intraspecific variation in colour patterns of ladybird beetles (Coleoptera: Coccinellidae) has been studied for many decades (Dobzhansky 1924, 1933; Lusis 1961; Majerus 1994; Majerus 1998). Generally, the elytra (the pair of forewings that are sclerotized in beetles) of ladybird beetles vary in the localization and extent of melanin, a dark pigment (Majerus 1998). In most species, there is a strong hereditary component determining melanism. There is temporal and geographic variation in frequencies of melanic individuals (Timofeeff-Ressovsky 1940; Creed 1975; Bengtson and Hagen 1977; Majerus and Zakharov 2000; Brakefield and de Jong 2011; Michie et al. 2011), and there is an ongoing debate about the nature of the selective forces maintaining colour polymorphisms in different species. Proposed theories include those related to thermal properties of black elytra ('thermal melanism') (Lusis 1961; Brakefield 1984c; Brakefield and Willmer 1985; De Jong et al. 1996; Michie et al. 2010), mimicry (Brakefield 1985), and mating preferences (Majerus et al. 1986). In the past two decades, the genetics underlying variation in melanism have been unravelled for several species of insects. Studies of *Drosophila* spp. have helped to decipher the melanin biosynthesis pathway, and the effector genes involved in this canonical cascade seem well conserved throughout the insects (True 2003; reviewed by Wittkopp and Beldade 2009; Van 't Hof and Saccheri 2010). The mechanisms responsible for regulation of the positioning of melanin in space and time, are however, far more diverse (Wittkopp et al. 2003; Van 't Hof et al. 2011), and have not been elucidated for ladybird beetles.

Winglessness in ladybirds

Some species of ladybird beetles also exhibit natural variation in wing length (Pope 1977; Hammond 1985; Majerus 1994). Little is known about the development and evolution of this trait (Pope 1977; Lommen et al. 2009), but interest has grown in the potential use of wingless morphs in biological control programs involving ladybirds as natural enemies (Lommen et al. 2008; Obrycki et al. 2009).

Melanism and winglessness in *A. bipunctata*

In some natural populations of the ladybird, *A. bipunctata*, both elytral colour pattern and elytron length are polymorphic (Lusis 1961; Marples et al. 1993) (Fig. 1). With respect to the colour polymorphism, hundreds of different colour morphs have been described, but three predominate in nature: the typical, non-melanic morph which has red elytra with two black spots (*typica*), and the melanic morphs with black elytra and with either four (*quadrimaculata*) or six (*sexpustulata*) red spots (Brakefield 1984a; Majerus 1998). It has been proposed that variation in elytra colouring is determined by a series of alleles at a major supergene, hierarchical in dominance, with melanic morphs generally dominant over non-melanics (Majerus 1998). However, the location of this supergene in the genome, and the sequence of the alternative alleles at this locus, are still unknown.

Typically, elytra cover the entire abdomen and the folded flight wings of *A. bipunctata.* In contrast, in wingless morphs, both pairs of wings (elytra and flight wings) are truncated, such that the abdominal part of the abdomen is not entirely covered by the elytra (Lommen et al. 2009). Wingless morphs are rare, but have been recorded for at least one population in The Netherlands (Marples et al. 1993), and five in the United Kingdom (M.E.N. Majerus, personal communication). So far, no fitness advantages have been found for the wingless morphs. The wild type is favoured for several life history traits (Ueno et al. 2004), and wild type females are more frequently mated by males than wingless ones (this thesis, chapter 7). Therefore, the potential adaptive value of winglessness in *A. bipunctata* in nature, if any, is unknown. Nevertheless, wingless *A. bipunctata* have been shown to be successful in biological control of aphids (Lommen et al. 2008; 2012). Winglessness (the presence of the truncation) is determined by one major, single locus with the winged, wild type allele being dominant over the wingless one (Marples et al. 1993). The location of the locus and the sequence of its alternative alleles, remain obscure. In this paper, we examine the degree of genetic linkage between these two wing traits, elytral melanism and winglessness, in *A. bipunctata* in classical genetic crosses.

The study population

Our observations on linkage between the wingless trait and elytral colour were all made in a laboratory population of wingless *A. bipunctata*. The origin of this population is described in detail by (2005). In summary, the ancestors of the population were all collected in the Utrecht region in The Netherlands, and one of them was wingless (homozygote recessive for the wingless trait). The frequency of the wingless alleles in the laboratory population was elevated to 100% by selecting wingless phenotypes in the F2 generation and breeding them together. To maintain a diverse genetic background, they were outcrossed to wild type beetles every now and then.

Figure 1. Variation in elytral colour and winglessness in *Adalia bipunctata*. Below each photograph, the corresponding phenotypes (P) and genotypes (G) are given, with letters representing the alternative alleles at both loci (M = melanic allele, m = typical allele, A = winged allele, a = wingless allele), with capital letters dominant over lower case letters.

Table 1. Linkage between the genes for elytral colour and winglessness in a laboratory population of *A. bipunctata*. Data are shown in separate rows for one group cross and for each of eleven individual families (fam). For the four largest families, the results are additionally split for sons and daughters. For each cross, the first grey block gives the parental genotypes, followed by the distribution of genotypes in the F1 offspring in the white block, with letters corresponding to the alleles (M = melanic allele, m = typical allele, A = winged allele, a = wingless allele), and capital letters dominant over lower case letters. The second grey block gives details on the linkage. Recombination fractions and map distances are only given for families with at least 50 offspring. G = value of G following a G-test of independence; df = degrees of freedom; P = probability value resulting from the G-test (significant p-values are indicated by an asterisk); RF = fraction of recombinant genotypes of the total offspring number; map distance according to Haldane's map function (in centiMorgans).

cross	parental genotypes		F1 offspring numbers per genotype (observed (expected))					genetic linkage				
	♂	♀	total	MmAa[a]	mmaa[a]	mmAa[b]	Mmaa[b]	G	df	P	RF	map distance (cM)
group	MmAa mmaa	mmaa MmAa	2426	1044 (778)	722 (456)	365 (631)	295 (561)	499.96	1	0.000*	0.27	38.8
fam 7	mmaa	MmAa	376	138 (102)	122 (86)	61 (97)	55 (91)	56.32	1	0.000*	0.31	48.0
			♂ *191*	*75 (57)*	*57 (39)*	*31 (49)*	*28 (46)*	*27.74*	*1*	*0.000**		
			♀ *185*	*63 (45)*	*65 (47)*	*30 (48)*	*27 (45)*	*28.01*	*1*	*0.000**		
fam 9	mmaa	MmAa	358	132 (99)	112 (79)	46 (79)	68 (101)	49.27	1	0.000*	0.32	50.6
			♂ *167*	*62 (47)*	*51 (36)*	*20 (35)*	*34 (49)*	*22.23*	*1*	*0.000**		
			♀ *191*	*70 (52)*	*61 (43)*	*26 (44)*	*34 (52)*	*27.21*	*1*	*0.000**		
fam 16	mmaa	MmAa	172	75 (52)	58 (35)	21 (44)	18 (41)	53.23	1	0.000*	0.23	30.2
			♂ *100*	*38 (23)*	*42 (27)*	*9 (24)*	*11 (26)*	*38.55*	*1*	*0.000**		
			♀ *72*	*37 (30)*	*16 (9)*	*12 (19)*	*7 (14)*	*13.41*	*1*	*0.000**		
fam 18	mmaa	MmAa	36	15 (10)	12 (7)	7 (12)	2 (7)	10.79	1	0.001*		
fam 25	mmaa	MmAa	82	26 (16)	35 (25)	7 (17)	14 (24)	20.89	1	0.000*	0.26	35.9
fam 28	mmaa	MmAa	27	9 (8)	7 (6)	6 (7)	5 (6)	0.90	1	0.342		
fam 29	mmaa	MmAa	170	69 (46)	62 (39)	19 (42)	20 (43)	52.37	1	0.000*	0.23	30.7
			♂ *87*	*33 (21)*	*34 (22)*	*10 (22)*	*10 (22)*	*26.79*	*1*	*0.000**		
			♀ *83*	*36 (25)*	*28 (17)*	*9 (20)*	*10 (21)*	*25.25*	*1*	*0.000**		
fam 32	MmAa	mmaa	27	12 (8)	10 (6)	4 (8)	1 (5)	12.70	1	0.000*		
fam 46	MmAa	mmaa	45	13 (9)	17 (13)	6 (10)	9 (13)	5.12	1	0.024*		
fam 50	MmAa	mmaa	24	8 (5)	9 (6)	5 (8)	2 (5)	4.85	1	0.028*		
fam 58	MmAa	mmaa	50	25 (20)	12 (7)	7 (12)	6 (11)	9.87	1	0.002*	0.26	36.7

[a]parental genotype
[b]recombinant phenotype

Detection of linkage between melanism and winglessness

We initially observed a correlation between melanism and winglessness while breeding the wingless population. We had composed groups of virgin, melanic, winged individuals (heterozygote for both traits) of one sex, mixed with virgin, typical, wingless individuals of the opposite sex (homozygote recessive for both traits). The F1 generation of these group crosses constituted the two parental phenotypes (typical, wingless individuals, and melanic, winged individuals), and the two recombinant phenotypes (melanic, wingless individuals, and typical, winged individuals) (Fig. 1). In total, 2426 offspring were scored for both traits. Since the wingless phenotype was less frequent than the winged one, expected frequencies of each of the four phenotypes were calculated based on allele frequencies in the total population of the F1 generation. 73% of the F1 offspring corresponded to one of the parental phenotypes, which is a significant deviation from the expected frequency, and suggesting linkage disequilibrium (Table 1). From these numbers, map distance was calculated to be 38.8 cM following Haldane's map function (Haldane 1919).

Subsequently in one of the outcrossing events, the wingless laboratory population was enriched with a further copy of a wingless allele. It originated from an individual found at the same locality that proved to be heterozygous for the wingless trait, and so all three alleles used to establish our stock were likely to be copies of the same wingless allele. We then used the enriched stock to confirm genetic linkage in similar crosses, but instead using twenty individual families. Eleven of them were bred from a melanic, winged male with a typical, wingless female, and nine from a typical, wingless male with a melanic, wingless female. All parents were virgin at the start of the experiment. Female ladybirds store sperm after mating, and lay eggs nearly daily throughout life (De Jong et al. 1998). F1 offspring were collected as long as the female laid fertilized eggs, bred to adulthood, and scored for elytral colour and winglessness. Offspring numbers varied widely from 4 to 376 with a median of 50 offspring. Because wingless *A. bipunctata* have lower longevity than winged ones (Ueno et al. 2004), the number of offspring of families with wingless mothers was lower than of those with a wingless father (Mann Whitney U-test, $U=19.5$, $P < 0.05$). Otherwise, there were no differences between these two types of families. Overall, nine families yielded too few offspring to allow statistical analysis and were, therefore, excluded. The results from the other eleven families are summarized in Table 1 showing offspring numbers per phenotype, the significance of genetic linkage calculated by G-tests of independences, and the estimated map distances. Ten of the eleven families showed significant linkage. A similar non-significant trend was seen in the other family. Four of the families were large enough to examine male and female offspring separately. All four demonstrated the same pattern for sons and daughters (Table 1), indicating that these loci are not on the sex chromosome. The nature of linkage was further calculated for the six families with at least 50 offspring. The recombination fraction ranged from 0.23 to 0.32 with an average of 0.27.

The map distance between the melanic and the wingless locus was estimated following Haldane's map function (Haldane 1919). It ranged from 30.2 to 50.6 cM with an average value of 38.7 cM, nearly exactly the distance initially estimated from the group crossing.

Conclusion & discussion: using linkage in molecular studies

In conclusion, we have shown that the major loci for winglessness and melanism of the elytra are genetically linked on one of the nine autosomes (Smith 1953) of *A. bipunctata*. Knowledge of this linkage may facilitate future molecular studies on the genetic bases of these two traits in *A. bipunctata*, even though the loci are not closely linked. A candidate gene approach can attempt to identify these two loci in *A. bipunctata*. Developmental studies on *A. bipunctata* have suggested that genes involved in dorso-ventral wing patterning provide candidates for winglessness (Lommen et al. 2009). Genetic tools, such as germ line transformation and RNA interference, are being developed for ladybirds (Kuwayama et al. 2006), and may further speed up such investigations.

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The rearing chamber with beetles in plasic dishes

5

Cryptic variation revealed - the genetic architecture of winglessness in the predatory ladybird beetle, *Adalia bipunctata*

Suzanne T. E. Lommen, Kees G. Koops, Bardo A. Cornelder, Peter W. de Jong, and Paul M. Brakefield

Abstract

Cryptic variation is standing genetic variation that does not contribute to the phenotypic range observed under standard conditions, but can be released under perturbing environmental or genetic conditions. We report on the genetic architecture of cryptic variation in wing length reduction in the ladybird beetle *Adalia bipunctata* (L.) which is only released in genetically 'wingless' morphs of this (otherwise winged) species. In this naturally wingless morph both pairs of wings are truncated. We established a wingless stock by outcrossing individuals carrying alleles for winglessness to wild-types from the same locality in The Netherlands. This stock, fixed for the recessive wingless allele, displayed wide continuous variation in the extent of wing reduction. Split-families reared at two temperatures revealed strong family-by-temperature interaction for this variation. Heritability was 0.64±0.09 at 19°C and 0.29±0.06 at 29°C. Artificial selection at 21°C demonstrated that the degree of wing reduction can be altered rapidly. A phenotype without any wing tissue, as well as one resembling the winged wild type, were obtained within a few generations by selection in opposite directions in two replicates. Analysis of frequency distributions in a pedigree covering three generations of selection demonstrated that the heritable component of the variation in wing reduction involves at least two polymorphic genes. We discuss the evolvability of this heritable cryptic variation in the wild, and its relation to the evolution of winglessness in other insect species. Finally, we argue that the manipulation of wing length can be exploited to improve biological pest control by wingless morphs of this ladybird beetle.

Keywords

Coleoptera: Coccinellidae, cryptic genetic variation, gene-by-environment interaction, modifier genes, insect wing development

Introduction

Cryptic variation

'Cryptic variation' is standing genetic variation that does not contribute to the range of phenotypes observed under standard conditions that natural populations experience, but can be 'released' and contribute to the phenotype under perturbing environmental or genetic conditions. The existence of such variation has long been recognized (reviewed by Schlichting 2008), and is common (e.g. Rutherford and Lindquist 1998; Sangster et al. 2008a). It has recently regained attention in relation to the developmental mechanisms that regulate its expression (reviewed by Rutherford 2000; Gibson and Dworkin 2004; Sangster et al. 2008b), and how it contributes to the evolvability of traits (Rutherford and Lindquist 1998; Rutherford 2003; Masel 2006; Le Rouzic and Carlborg 2008; McGuigan and Sgro 2009; Hayden et al. 2011).

Cryptic variation contributes to wing reduction in flightless morphs of *Adalia bipunctata*

Here, we describe the genetic architecture of cryptic variation in the two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), that is revealed in a natural 'wingless' morph of this species (Marples et al. 1993). Wild-type beetles are fully winged and monomorphic in their wing length, with elytra (fore wings) covering the entire dorsal surface (Fig. 1). Their flight wings (hind wings) are folded underneath the elytra when at rest, but are nearly twice as long as the elytra when unfolded in flight (Lommen et al. 2009). 'Wingless' morphs are occasionally encountered in the wild (Majerus and Kearns 1989; Marples et al. 1993). They have been recorded from at least one population in The Netherlands (Marples et al. 1993), and five in the United Kingdom (M.E.N. Majerus, personal communication). Their elytra and flight wings are truncated (Lommen et al. 2009) so that they cannot fly. Although they may have some wing tissue, we will for ease, call them 'wingless'. This trait is determined by a major locus with the winged, wild-type allele being dominant over the wingless one (Marples et al. 1993; Ueno et al. 2004) (Fig. 1). Since only a few wingless specimens from the wild have been reported on in detail, the variation within the wingless phenotype in the wild is unknown. However, several wingless stocks have been established by breeding from a single wingless individual taken from the wild near Utrecht in The Netherlands and crossed with wild types from the same locality. In each case, the wingless F2 individuals exhibited wide variation in the extent of the wing reduction (Marples et al. 1993; Ueno et al. 2004; Lommen et al. 2009). This variation is continuous from individuals lacking all wing tissue to ones missing only very small pieces at the wing tips (Fig. 1). This variation is associated with fitness traits since those with more marked reductions develop more slowly and have a shorter life span (Ueno et al. 2004). Earlier work suggests that the extent of wing reduction is a result of differences in the rate of wing development in the larval stages, and is regulated by both genetic and environmental

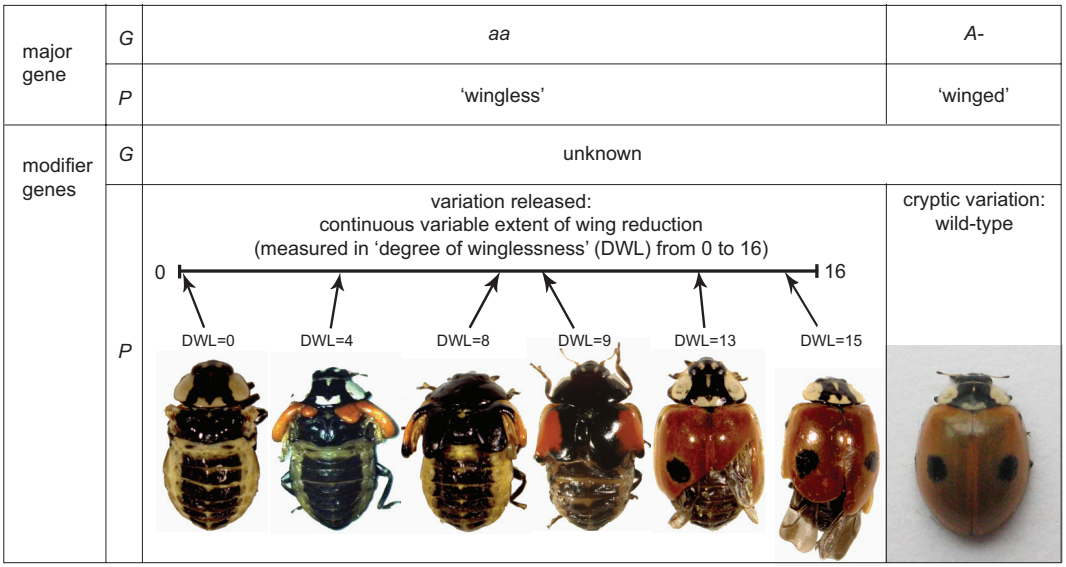

Figure 1. Genetic (G) and phenotypic (P) variation in wing morphology in *Adalia bipunctata.* The upper part of the graph covers the major gene regulating winglessness, the lower part shows the modifier genes regulating the extent of wing reduction in wingless morphs. This extent is expressed in the degree of winglessness of the elytra (DWL), a categorical measure with values ranging from 0 (no wing tissue) to 16 (elytra covering more than ¾ of the abdomen, see materials and methods for details). The pictures of the six *wingless* specimens represent examples of particular DWL categories. The middle two individuals are *melanic* colour morphs, whereas the others are *typical* morphs. A *wild-type* (winged) typical ladybird beetle is shown at the right.

factors (Ueno et al. 2004; Lommen et al. 2005; Lommen et al. 2009). Since all wingless beetles are homozygous recessive for the wingless trait, the heritable variation in the extent of wing reduction within wingless morphs is determined in the genetic background that reflects the standing genetic variation in the almost completely winged wild population. Thus, variation in the extent of wing reduction is cryptic in the wild-type phenotype, whereas it is released in the wingless phenotype.

The significance of this cryptic variation

The significance of this cryptic variation in natural populations of *A. bipunctata* is unclear, since wingless individuals are scarce and the potential for pleiotropic effects on fitness traits in wild types is unknown. Nevertheless, the variation in the extent of wing reduction in wingless adults is of interest in this system for several reasons. Firstly, winglessness is a derived trait that is common in beetles (e.g. Darlington 1936; Smith 1964; Dybas 1978; Hammond 1985), and in many other insect groups (Wagner and Liebherr 1992; Roff 1994a). The mechanisms of development and evolution of wing reduction are, however, diverse and not fully understood (e.g. Roff 1994b; Abouheif and Wray 2002; Brisson et al. 2010). Typically, wing polymorphic populations exhibit discrete winged and wingless phenotypes that each show little variation in wing morphology. Only in a few cases

has variation in the extent of wing reduction within the 'wingless' morph been recorded in nature. These include examples in beetles (Darlington 1936; Den Boer et al. 1980; Desender 1989), and stoneflies (Plecoptera) (McLellan 1999). Such variation might provide a key to understanding instances of the evolution from entirely winged populations to those including wingless morphs. Secondly, there is interest in the use of wingless morphs of ladybird beetles as natural biological control agents of insect pests in agriculture (Lommen et al. 2008; 2013). Understanding the basis of variation within the wingless morph, including the potential for its manipulation, may help to optimize this morph for its application in biocontrol.

In this paper, we investigate in detail the genetic architecture of variation in the degree of wing reduction in wingless *A. bipunctata*. We quantify the heritability of the degree of wing reduction, demonstrate that we can change this rapidly by artificial selection, and examine genetic models that can explain observed patterns of segregation using a pedigree approach.

Materials and methods

Beetle laboratory stock

A laboratory stock of wingless *A. bipunctata* was established from individuals collected in the wild in Utrecht in The Netherlands on several occasions. Beetles were collected from trees or shrubs. Two individuals were found to bear copies of the wingless allele, one homozygote and one heterozygote. The three founding wingless alleles are assumed to be identical by descent (see further below). The wingless allele was fixed in the laboratory stock by outcrossing these beetles to more than 100 wild types and then selecting only wingless phenotypes as parents in the large F2. This stock was then used for all experiments. It is maintained at 20.5°C ± 1.5°C, RH 65% ± 5%, and 16L:8D. Larvae and adults are fed with *Ephestia kuehniella* Zeller eggs, with adults receiving a supplement of flower pollen (De Clercq et al. 2005).

Measuring wing reduction

We used the measure 'degree of winglessness' (DWL) to quantify the continuous variation in wing reduction of wingless *A. bipunctata* (see also Lommen et al. 2005). The degree of the truncation of the elytra is closely correlated with that of the flight wings (Lommen et al. 2009). The latter are not visible when folded beneath the elytra and, therefore, DWL is based on the length of the elytra alone. DWL indicates the length of the truncated elytra relative to the maximum length as would be recorded in the wildtype where the elytra cover the entire abdomen. Because the left and the right elytron, and even the medial and outer half of a single elytron, may be truncated to different extents, the length of each of these four halves is scored separately. The length of each

of the four elytral sections was estimated by eye and assigned 0 (no tissue visible), 1 (only a small bud, not extending beyond ⅛ of the maximum), 2 (up to ¼ of the maximum), 3 (more than ¼ and up to ½ of the maximum), 4 (more than ½ and up to ¾ of the maximum), or 5 (more than ¾ of the maximum). The sum of these four scores is an individual's DWL, with a minimum value of 0 and a maximum of 20, with higher numbers corresponding to increased elytron length (Fig. 1). This method is highly repeatable (Lommen et al. 2005). However, here it proved problematic for statistical analysis because the range of DWL categories is not symmetric: more than half of the DWL categories represent tissue covering up to only half of the maximum tissue length. Therefore, we modified the calculation of DWL by combining score 1 (only a small bud, not extending beyond 1/8 of the maximum) and 2 (up to ¼ of the maximum). As a result, the new summed DWL value (the sum of the four scores) became a symmetric range from 0 to 16 (Fig. 1). Individuals with a DWL of 0 have no elytral tissue, whereas, a DWL value of 16 was assigned to beetles with all four elytral parts extending beyond ¾ of the maximum elytral length (note that morphological variation remains within this latter category).

Heritability experiment

A series of families of wingless *A. bipunctata* were bred to estimate the heritability of DWL by parent-offspring regression. In addition, the offspring of each family were split and reared at two different temperatures to examine environmental effects. Three-week old virgin adults from the stock were used to set up pairs. We first selected individuals with different DWL values to maximize variation in wing reduction in the parents. We then formed pairs using males and females with nearly equal DWL values. In this way the range of mid-parent values was maximized to increase the reliability of the heritability estimate (Falconer and Mackay 1996). Pairs were kept in round Petri dishes (Ø 55 mm) with paper lined in the cover to stimulate egg-laying under our rearing conditions. These papers were collected and replaced every 2-3 days over a period of 12 days. Collected papers with eggs were moved to a clean dish. Hatched first instar larvae were again moved to a clean dish and reared in groups of up to 10 until moulting. After moulting, the second instar larvae were randomly assigned to an incubator with one of two rearing temperatures, 19°C or 29°C, and within which their position was regularly changed. Histological examinations show that the formation of wing structures does not begin until this larval stage or later (Lommen et al. 2009). Emerged adults were frozen at -20°C before their sex and DWL were determined within several weeks. We selected for analysis those families with at least five offspring at each rearing temperature (N = 41 families, median = 15 and 12 offspring at 19°C and 29°C, respectively).

Statistical analysis used the package R, version 2.6.1 (R Development Core Team 2007). The mid-offspring values were represented by the median DWL of each family to correct for non-normal frequency distributions. We pooled sons and daughters since sample sizes per sex were small

whereas the variation among offspring was large, and because earlier work revealed no differences between the sexes (Lommen et al. 2005). The heritability experiment was analysed using linear models fitted by least square methods using weighting by the number of offspring. In the full model, family, temperature, and their interaction term were specified as fixed factors. We tested the significance of the interaction by removing this term from the model and comparing the models by an F-test. The heritability of DWL was then estimated for each rearing temperature separately as the mid-offspring on mid-parent regression.

Artificial selection experiment

An artificial selection experiment was performed to estimate the realized heritability of DWL in our stock at 20.5°C and 65% RH. We selected in both upward and downward directions using two replicates. Generation 0 (G0) was derived from the wingless stock after artificial selection for increased fecundity over ten generations (S. T .E. Lommen, K. G. Koops, P.W. de Jong., and P. M. Brakefield, unpublished data). We randomly split this population in half to create the two replicate lines: 'C' (original N=1136 virgin beetles) and 'D' (N=1126). For each replicate, we selected 60 males and 60 females with the most extreme values in each direction: 'L' and 'H' for downward and upward selection, respectively (yielding lines: 'CL', 'CH', 'DL', and 'DH'; Table 1). For each line, the selected adults were randomly distributed into three breeding groups, each of 20 males and 20 females, at an age of 6-26 days when most beetles are sexually active (Hemptinne et al. 2001). Breeding groups were kept in Petri dishes (120 mm x 120 mm) with paper folded like a harmonica to stimulate egg-laying. Eggs were collected every 2-3 days over two weeks (except for G4, over four weeks) and reared to adulthood. Newly emerged adults were collected every 2-3 days and housed individually in small dishes (Ø 55 mm) until after determination of their sex and DWL to ensure that all adults remained virgin until after selection. Occasionally when insufficient offspring were bred, fewer than 60 adults per sex were collected (as in G2 and G3 of line CL; Table 1). When the most extreme DWL value in the direction of selection contained more than 60 adults per sex, all of them were selected as parents (Table 1). Selection was continued for four generations, after which an extra round of selection was performed for the upward selected lines to check whether the potential remained for a further response towards an elytral phenotype even closer to that of the wild type. The phenotype of beetles with a DWL of 16 for a subset of the G4 (see Table 1) and for the entire G5, was described in more detail by recording whether the elytra covered the full length of the abdomen. All beetles selected as parents in G4 for the extra round of selection had a DWL of 16 and were of this latter phenotype.

To analyze the results, we first tested whether DWL differed between the sexes for each selection line at each generation. We used a Wilcoxon Signed Rank Test with continuity correction, adjusting the alpha with a Holm-Bonferroni correction. Since no significant differences were detected, we

Table 1. Numbers of bred and selected adults per generation for each selection line

replicate	direction of selection	line		number of adults in generation					
				0	1	2	3	4	5
C	downward	CL	bred:	1136[a]	464	42	35	156	NA
			selected:	120	133	39	32	NA	NA
C	upward	CH	bred:	1136[a]	552	785	606	519[b]	881
			selected:	120	120	120	160	120	NA
D	downward	DL	bred:	1126[c]	329	292	133	99	NA
			selected:	120	122	149	102	NA	NA
D	upward	DH	bred:	1126[c]	710	455	547	777[d]	781
			selected:	120	119	120	160	120	NA

NA=not applicable

[a] CL and CH were selected from the same group of G0 adults

[b] for a subset of 320 beetles, elytra of those with DWL=16 were described in more detail

[c] DL and DH were selected from the same group of G0 adults

[d] for a subset of 577 beetles, elytra of those with DWL=16 were described in more detail

pooled the sexes for further analysis. A comparable procedure was followed to examine differences in DWL between the low and high line of each pair of replicate lines in each generation. We then estimated the realized heritability of the DWL for each selection line as the slope of the regression of the cumulative response to selection (median offspring value - median of the total parental population) on the cumulative selection differential (median of the selected parents - median of the total parental population). We calculated the mean realized heritability for each direction of selection by averaging the values of the two replicates. A heterogeneity G-test (Sokal and Rohlf 1995) was used to test whether the proportion of beetles with a DWL of 16, and with elytra covering the full length of the abdomen, had changed after the extra round of selection in the upward selection lines. Finally, a 1-tailed paired t-test was used to test the hypothesis that the average number of offspring per selected adult was higher for the lines selected in the upward than in the downward direction for each of the replicates.

Pedigree analysis

To study the genetic architecture of wing reduction in wingless *A. bipunctata* in more detail, we created a pedigree over four generations, and then examined whether genetic models could be fitted to the data. We used G4 of each of the four selection lines to set up the pedigree as shown in Figure 5a. Each family was bred from a pair of virgin beetles. We first set up pairs within each selection line with similar DWL values (P). In the next generation (F1), we crossed individuals descending from opposite selection directions within each replicate (i.e. CL x CH and DL x DH). In addition, we created inter-line crosses between the replicate high lines (CH x DH). Offspring from

these crosses (F2) were then interbred within their own group to produce the F3 families (parents always came from different families to minimize inbreeding). We determined the sex and DWL of all individuals raised and only included families with more than 20 offspring in the analysis (except for one smaller, but essential, family) yielding a total of 96 families. Since very rapid responses to artificial selection occurred (see results), we hypothesized that DWL is regulated by a small number of genes and begun our analysis using a model of one polymorphic gene.

Data archiving

The data sets resulting from each of these experiments will be deposited in the public, digital archive Dryad (www.datadryad.org).

Results

Heritability experiment

The degree of winglessness (DWL) of the parents ranged from 0 to 12, since pairs with higher DWL values produced insufficient offspring, and were excluded from the analysis. There was a significant family-by-temperature effect ($F=9.98$, $p<0.01$). Raising larvae at higher temperatures usually yielded lower median DWL values (Fig. 2), with this pattern being observed in 35 of 41 families. The mean heritability was estimated as 0.64 ± 0.09 (SE) at 19°C ($F_{1,39}=53.35$, $p<0.001$, $R^2=0.58$) and 0.29 ± 0.06 (SE) at 29°C ($F_{1,39}=23.00$, $p<0.001$, $R^2=0.37$) (Fig. 2).

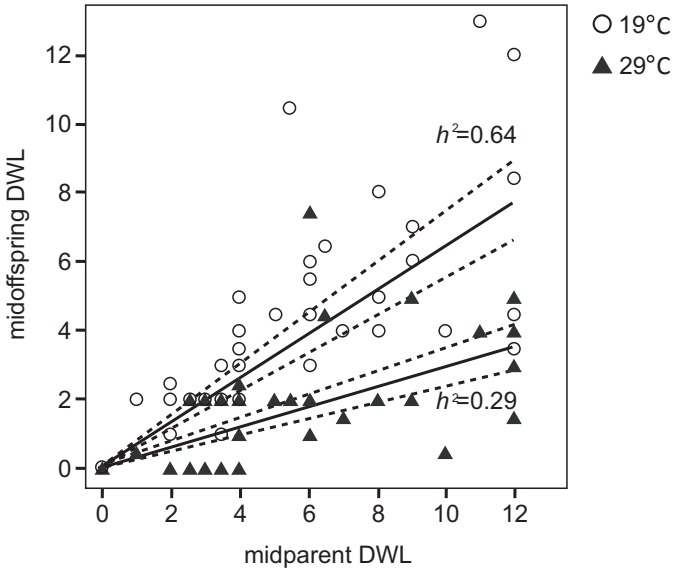

Figure 2. Heritability of DWL. The degree of winglessness (DWL) plotted as the midoffspring on midparent values for split families raised at two temperatures. The slope of the regression lines (shown between 95% confidence intervals) indicates the heritability (h^2) of DWL estimated for each temperature, and significantly differs between the two temperatures.

Artificial selection experiment

Males and females of G0 did not significantly differ in DWL (N_{males}=1130, $N_{females}$=1132, W=615508.5, p=0.118). An absence of a sex effect was also shown after the population was split into replicates and selected for one generation (CL: W=28037.5, p=0.337; CH: W=35214, p=0.362; DL: W=12799, p=0.3752; DH: W=67930, p-value=0.070), and in all of the subsequent generations of all four selection lines (not shown). A rapid response to artificial selection on DWL was observed in the replicate lines in each direction (Fig. 3). In both replicates, the upward and downward lines differed significantly from each other in DWL after only a single generation of selection (C: W = 17831.5, p<0.000; D: W=13480, p<0.000). The response to selection continued until in both downward selected lines, the median DWL reached the minimum possible value (DWL=0) after only two generations, whereas that of the upward selected lines both took four generations to reach the maximum score (DWL=16). Corresponding realized heritabilities were 0.72 ± 0.07 and 0.61 ± 0.03 for downward and upward selection, respectively (Fig. 4).

Although the majority of the beetles in the upward direction had reached this maximum score after four generations of selection, the proportion of individuals with elytra covering the full length of the abdomen was only 48% and 35% in CH and DH, respectively. It is noteworthy that the majority of these latter beetles remained distinct from wild types because of irregularities in the three-dimensional shape of their elytra.

After the extra selection round of selection in which all parents had elytra covering the full length of the abdomen, the proportion of individuals with a DWL of 16 significantly increased from 65% to 70% in replicate CH (G_H=3.845, p<0.05) and from 50% to 71% in DH (G_H=68.216, p<0.001). The proportion of individuals with elytra covering the full length of the abdomen did not change significantly in line CH (G_H=2.297, p=0.130; 48 to 43%), whereas it increased from 35% to 46% in line DH (G_H=16.493, p<0.001).

In line D, the number of offspring produced was significantly higher in the upward than in the downward direction of selection (t=5.36, df=3, p<0.01). The same trend occurred in line C but was not significant (t=1.33, df=3, p=0.138).

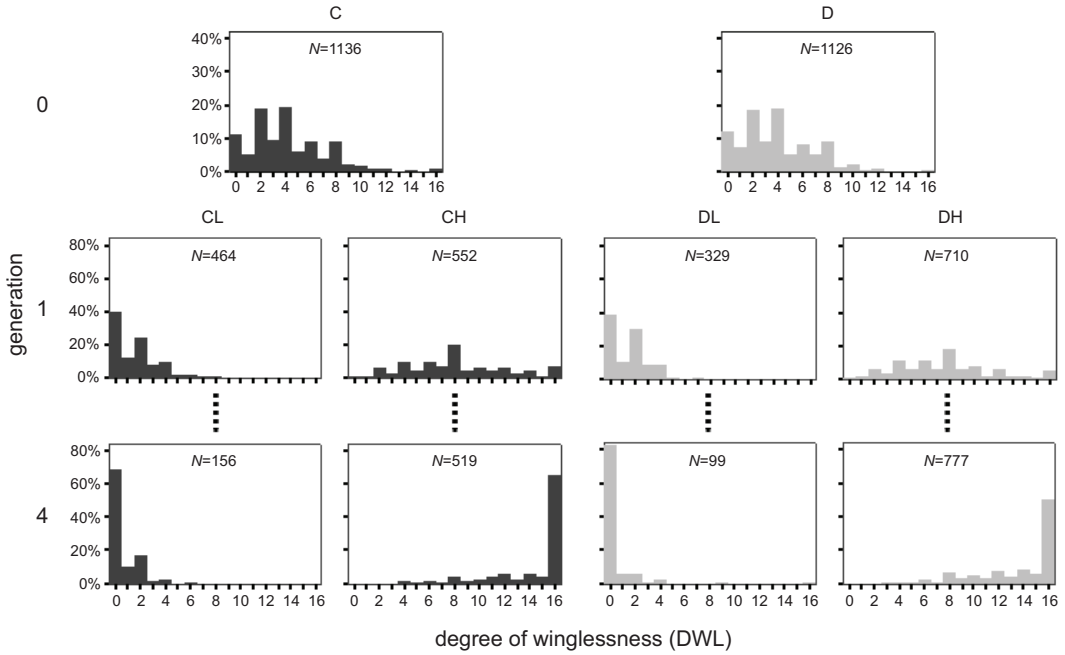

Figure 3. Artificial selection for extreme DWL. Frequency distributions of two replicate lines (C and D) artificially selected in downward (L) and upward (H) directions for degree of winglessness (DWL). Bars in histograms represent percentages for each DWL class, whereas total sample sizes are indicated for each histogram. The initial subpopulations (generation 0) are shown, together with the populations one, and four generations after selection began. There is a strong response to selection, and after four generation the majority of the beetles has reached the maximum value in the selected direction. The replicates show a similar pattern.

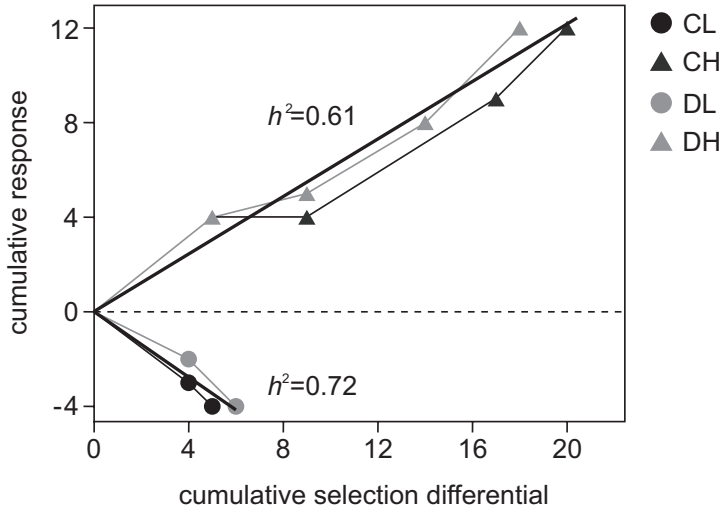

Figure 4. Realized heritabilities of DWL. Cumulative response to artificial selection as a function of the cumulative selection differential for both replicates (C, D) selected in upward (H) and downward (L) directions for degree of winglessness (DWL). Mean realized heritabilities (h^2, thick solid lines) are given for each selected direction.

Pedigree analysis

We examine here a representative part of the entire pedigree that comprises 15 families over three generations (data from other parts of the pedigree yielded consistent interpretations; the full data set is available on request). Using 15 families (Fig. 5b), we first demonstrate that variation in DWL is unlikely to be regulated by a single polymorphic locus with two alleles before moving on to more complex models. In the context of a single-gene, bi-allelic model we can make several general predictions with respect to the parental genotypes used to found the pedigree from the selection lines at G4 (generation P). Given that the lines showed progressively less variation with each generation of selection, and that those selected in opposite directions displayed very little overlap in phenotypic distribution after four generations (Figure 3), we assume that individuals from opposite selection lines are near fixation for alternative alleles at this single gene, and that the majority of them are homozygous. Since the estimated heritabilities of DWL were high, variation in wing reduction appears to be mainly additive and we assume no dominance or epistatic interactions. Under these assumptions, we can predict that the frequency distributions of DWL of the offspring are: 1) different for F1 families from lines selected in opposite directions; 2) similar for F1 families from lines selected in similar directions; 3) equal across all F2 families, regardless of parental origin, and consist exclusively of heterozygotes; and 4) equal across all F3 families which would constitute each of the three possible segregating phenotypes, and would therefore have a wider range of phenotypes than the F2.

We can then compare these predicted phenotypic distributions to those observed in the 15 families shown in Figure 5b and all descended from lines DL and DH. Phenotypes of the parents (DWL values) are given at the top of each histogram (note that all founding parents had extreme DWL values of either 0 or 16, increasing the likelihood that they were homozygote under the assumptions above). Prediction 1 fits the results since F1 families from opposite lines indeed differ in their frequency distributions (DL: family 2 and 4, versus DH: family 1, 3, and 5). However, the remaining predictions do not fit the pedigree. Prediction 2 is rejected because F1 families from DH display variation in the frequency distribution (e.g. family 3 versus 5). Prediction 3 does not hold because the distributions are variable across families within generation F2 (compare families 6-7 with 8-10). Prediction 4 does not fit the observed data because distributions of F3 families differ from each other (families 11-15), do not cover a wider range than the F2 families, and do not all include all three possible segregating phenotypes under the bi-allelic model (e.g. family 13 lacks high DWL values). Even allowing for heterozygosity in some founding individuals to explain variation across the F1 families, the model cannot explain patterns of variation in the F2 and F3 families. Hence, we reject a single-gene, bi-allelic model for the control of wing reduction.

We then explore a model of a single gene with multiple alleles, and also allow founding parents to be heterozygous rather than homozygous. However, this model fails to explain the absence of some

phenotypes in F2 and F3 (e.g. families 6, 7, and 13 lack high values). Thus, the pedigree reveals that a single polymorphic gene, regardless of the number of alleles at this locus, cannot explain the observed variation of wing reduction within the wingless morphs, and we conclude that variation in wing reduction is regulated by more than one locus (i.e. polygenic). With this conclusion, we implicitly reject the possibility that variation is explained by an allelic series of several wingless alleles at the major wingless locus.

A model with two bi-allelic genes with full dominance but without epistatic interactions also failed to explain the data because two parents with low values of DWL (corresponding to homozygote recessives at one of the two genes) were able to produce the full range of phenotypes (including phenotypes corresponding to dominant alleles on this gene) (e.g. family 14, Figure 5) . Therefore, we also reject this model and conclude that a more complex one is required. Such a model could involve more genes, more than two alleles at some genes, or epistatic interactions (note that reciprocal crossings give no evidence for paternal or maternal effects, e.g. family 6 versus 7).

Discussion

The genetic architecture of wing length variation in *A. bipunctata*

Our results show that the variable extent of wing reduction in wingless morphs of *A. bipunctata* is highly heritable (Fig. 2 and 4), and that there are gene-by-environment interactions (Fig. 2). The degree of winglessness changed rapidly under artificial selection over four generations (Fig. 3). These results suggest that few loci with large effects are involved. The analysis of the pedigree (Fig. 5) shows that the inheritance of the extent of wing reduction in our stock is polygenic, and that these genes are likely to be multi-allelic. None of the experiments or the pedigree indicates any genetic linkage between these genes and sex.

Thus, we can now present a more complete model of the genetics of winglessness in this species. This is likely to involve at least three polymorphic loci on the autosomes regulating wing morphology: one determining the wingless status (Marples et al. 1993, upper part of Table 2), and at least two multi-allelic modifier loci that are affected by the environment and regulate the variation in the extent of wing reduction within the wingless phenotype (this paper, lower part of Table 2).

In the artificial selection experiment, an extra round of selection in the upward direction resulted in an increased proportion of individuals in the highest category, DWL=16, but only one of the two replicates resulted in more beetles with elytra covering the full length of the abdomen. This suggests that at least one polymorphic modifier locus had not yet reached fixation.

Since all wingless individuals in our experimental stock are homozygous recessive at the wingless locus, our results confirm that the expressivity of the wingless mutation is dependent on the

Table 2. A genetic model suggested for natural wing polymorphism in *A. bipunctata*. The table provides the number of genes involved (N), the number of alternative alleles per gene, the position of the genes, the heritability and expression of the trait regulated. The upper part of the table provides details on the major gene regulating winglessness, the lower part on modifier genes associated with variation in the expression of this trait.

	regulating	N	alternative alleles	position	heritability	expression	sources
major gene	wingless-ness	1	2	on an autosome, linked with gene for melanism	1.0	**dominance:** 'winged' completely dominant over 'wingless' **epistasis:** 'winged' allele shows full penetrance and dominant epistasis to the modifier genes	Marples *et al.* 1993; Lommen et al. 2012
modifier genes	extent of wing reduction	≥2	≥2 at each locus	on autosomes	**parent-offspring regression:** - at 19°C: 0.64±0.09 - at 29°C: 0.29±0.06 **realized h^2** at 21°C**:** - upward: 0.61±0.03 - downward: 0.72±0.07	**conditional:** - when major gene has at least 1 'winged' allele: no visible phenotype (cryptic) - when major gene is homozygous for 'winglessness': expression dependent on *G*x*E* interaction (variation 'released')	Ueno et al. 2004; Lommen et al. 2005; this paper

genetic background (Ueno et al. 2004; Lommen et al. 2005; 2009). The latter reflects the standing genetic variation at the field location, which has no visible phenotypic effect on wing morphology in the winged phenotypes (Lommen et al. 2009). This cryptic variation could have evolved by the accumulation of neutral mutations that scarcely, if at all, affect traits under selection in the wild type. Alternatively, it could have positive pleiotropic effects on such traits under selection, and have evolved by stabilizing selection on these traits (e.g. Duveau and Felix 2012). However, the substantial amount of heritable variation for the degree of winglessness, as demonstrated in our study, suggests that this variation is neutral with respect to fitness traits. Genetic canalization, where wing development is robust against genetic mutations, may be a mechanism explaining its cryptic nature (Waddington 1957; but see Hermisson and Wagner 2004; Zhang 2008).

Although cryptic variation is hard to detect, especially in nature, this phenomenon has been recognized in several other complex traits in model organisms. Examples include sense organs and body appendages of *Drosophila melanogaste*r (Gibson and van Helden 1997; Polaczyk et al. 1998; Atallah et al. 2004; Dworkin et al. 2009), diseases in mice and humans (Nadeau 2001), and sexual characters in *Caenorhabditis elegans* (Milloz et al. 2008; Chandler 2010). Recent studies in these organisms show that background effects can have substantial phenotypic consequences as large as those of known mutations in major genes (Gibson et al. 1999; Atallah et al. 2004). Our artificial

selection experiment similarly demonstrates that the wingless phenotypes in *A. bipunctata* span the range from those without any wing tissue to those indistinguishable from the wild type.
Our results confirmed that there is a family-by-temperature effect on the extent of wing reduction in *A. bipunctata*, providing evidence that the genetic background and the environment interact to produce the phenotype. Some other studies have also revealed cryptic genetic variation that interacts with the environment highlighting how a complex interplay between these factors may regulate the development of complex adult phenotypes, and that understanding such interactions will be crucial in explaining phenotypic diversity (Atallah et al. 2004; Chandler 2010). A challenge for future research is to examine the evolution of cryptic variance, and its consequences for the evolvability of traits in natural populations (Le Rouzic and Carlborg 2008; McGuigan and Sgro 2009).

Evolvability of the cryptic variation in the extent of wing reduction

Our artificial selection experiment demonstrates that the cryptic variation regulating the extent of wing reduction is evolvable in a laboratory environment. Wingless phenotypes showing little variation were obtained in each direction of selection. Thus, the downward direction yielded individuals consistently without any wing tissue, whereas a phenotype closely similar to the wild type resulted in the upward direction effectively masking the wingless genotype with respect to elytra morphology.

Restoration of the wild-type phenotype including flight ability

The extent to which the flight ability of beetles homozygous for the *wingless* allele can be restored remains to be investigated. Histological examinations of the flight muscles of wingless morphs did not reveal any reduction in muscle tissue (S. V. Saenko and S. T. E. Lommen, unpublished results), and wingless individuals were frequently observed to move their truncated wings up and down in a similar manner as wild types (S. T. E. Lommen and K. G. Koops, unpublished results). The flight wings of wingless individuals were usually reduced or malformed (Lommen et al. 2009), and the beetles were typically not capable of directed flight. However, observations on flight behaviour suggest that the potential for at least some flight activity was restored in a small proportion of wingless individuals after artificial selection in an upward direction (S. T. E. Lommen, K. G. Koops, P. W. de Jong, P. M. Brakefield, unpublished data). Thus, when five random samples of ten G4 beetles each from the upward selection lines were given the opportunity to fly up from vertical sticks surrounded by a water barrier, several individuals attempted to fly by opening their elytra and spreading their flight wings, but only a few of these managed to take off (of which only a single individual flew up substantially, more than a meter in height), and all of them quickly dropped down or landed in the barrier. In contrast, from one group of ten wild types, five flew up and away passing

the water barrier, and a further two flew up and landed in the water barrier. In another flight test where phenotypes with elytra both in length and in three-dimensional shape indistinguishable from the wild type were released in the air, only three out of 17 beetles flew in a directed manner, whereas 16 out of 18 wild types did.

Such evolution towards a wild-type phenotype has also been observed in the laboratory for the eyeless-4 mutant of *D. melanogaster* (Spofford 1956). Isogenic populations, homozygous recessive for the eyeless trait, displayed variable reduction in eye size which was negatively correlated with reproduction, and mean eye size moved towards that of the wild type eye size over several rounds of selective breeding. Several mechanisms could account for such a recovery of wild-type phenotypes including selection of modifier genes that diminish expression of the mutant phenotype through epistasis (Gibson and Dworkin 2004), selection of an epigenome that buffers the effect of the mutation in the major gene (Sollars et al. 2003; Johannes et al. 2008), or selection for modifiers that produce the wild type phenotype via another route in the genetic regulatory network (Tautz 1992; Kitami and Nadeau 2002).

Evolution of wing reduction in the wild

How likely is such evolution of this cryptic variation in *A. bipunctata* in nature? It is unlikely to evolve in the wingless phenotype of *A. bipunctata*, since it is rare in the wild and no long-term adaptive value of winglessness seems very likely in this species. Thus, wingless individuals show reduced fitness traits related to development time, life span, and reproduction compared to wild-type beetles (Ueno et al. 2004), and wingless females are less frequently mated with in the laboratory (S. T. E. Lommen, E. V. Bitume, P. W. de Jong, P. M. Brakefield, unpublished data). However, cryptic variation may evolve in a wild type phenotype when this variation has pleiotropic effects on fitness traits (Duveau and Felix 2012). This could be examined in our system by introgressing the winged phenotype into the wingless lines artificially selected for low and high degree of winglessness.

Evolvability of wing reduction and evolution of wingless insect morphs

The evolvability demonstrated in our artificial selection experiment is of interest in the context of the evolution of wingless morphs in other insects. In species where winglessness is an adaptive trait in nature, the wingless phenotypes are typically monomorphic and typically lack any wing tissue. One model for the evolution of such extreme wingless phenotypes is a gradual reduction of wing tissue over time as has been suggested for carabid ground beetles (Den Boer et al. 1980), wingless populations of Hawaiian brown lace wings (Tauber et al. 2007), and the wingless castes of ants (Nahmad et al. 2008). Our study shows there are no developmental constraints to the production of a continuous phenotypic range in the degree of wing reduction in a laboratory stock of *A. bipunctata*, and that intermediate phenotypes can be readily produced in this species. We have

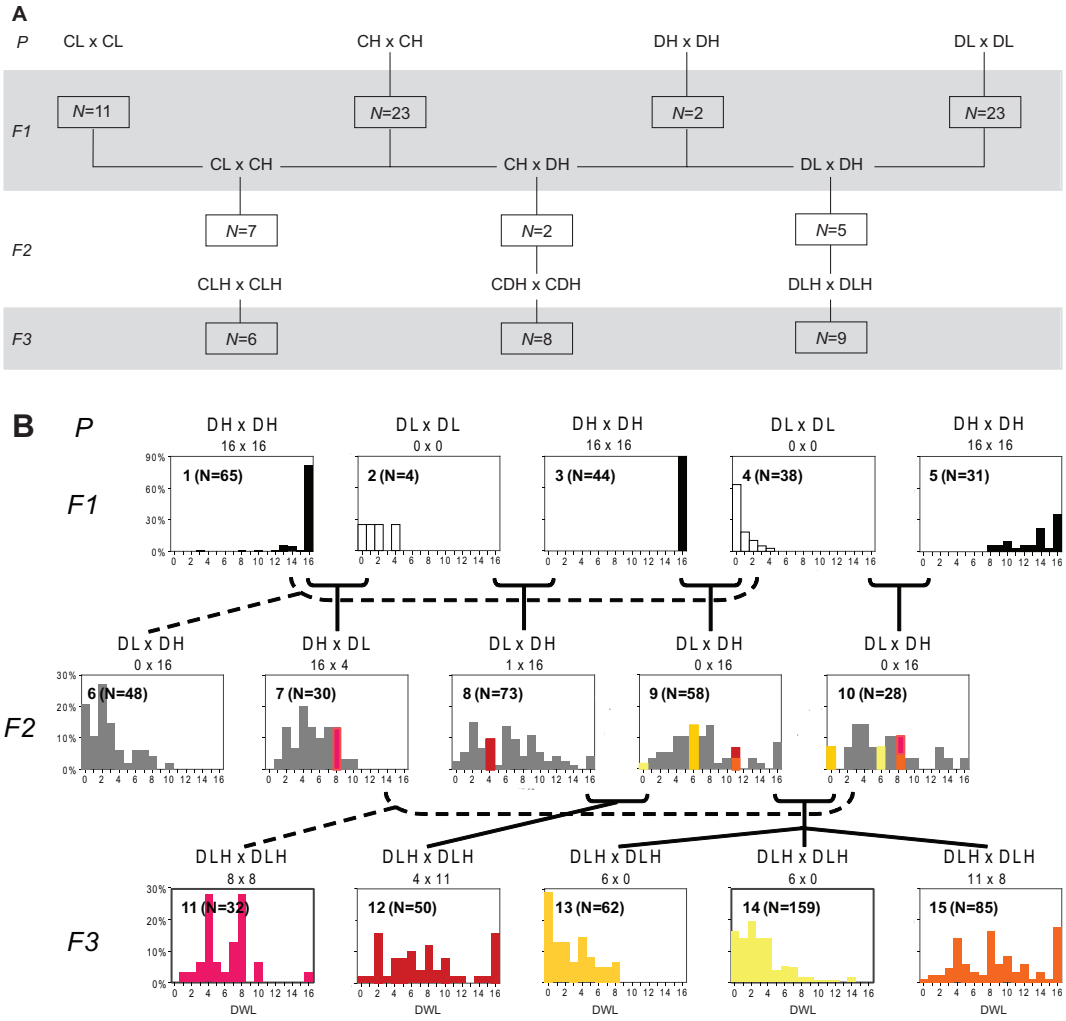

Figure 5. Pedigrees established from beetles artificially selected for extreme degrees of winglessness. (A) Scheme of the entire pedigree set-up, comprising generations *P*, *F1*, *F2*, and *F3*. C and D are replicate lines from the artificial selection experiment, and L and H indicate downward and upward directions of selection (altogether giving four selection lines: CL, CH, DL, and DH). *N* is the number of families bred; only 1-4 of these were used as sources for the next generation. (B) A part of the pedigree constituting fifteen families descending from line DL and DH. Histograms are numbered 1-15, and show frequency distributions of the degree of winglessness (DWL) in percentage values with family sizes (*N*) in brackets. The first line above each histogram indicates the type of cross corresponding to the scheme in Figure part A, whereas the second line gives male (left) and female (right) parent DWL. Note that the scale of the y-axis of the *F1* families differs from that of the subsequent generations. *F1* families from DL and DH are indicated in white and black, respectively. *F2* families (all resulting from a DL x DH cross) are presented in grey, with the categories of those individuals selected as parents of the *F3*, each marked in the colour of the corresponding *F3* family they produced. The pedigree suggests that at least two polymorphic modifier genes regulate DWL.

demonstrated that such cryptic variation can include a heritable component that natural selection could act upon. Therefore, even though winglessness is unlikely to be adaptive in *A. bipunctata*, our study illustrates the potential for gradual evolution. The cryptic variation released within the 'wingless' morph in our study could mimic a transitory evolutionary phase from an ancestral monomorphic winged population towards a dimorphic population with discrete phenotypes, or alternatively a monomorphic wingless species.

Evolvability of wing reduction in wingless A. bipunctata and use in biological control

Finally, the evolvability of the degree of wing reduction in *A. bipunctata* could be exploited for their application in biological control of aphids in crops or in trees in urban environments. The flight dispersal of adults is considered to reduce ladybirds' effectiveness in commercial releases (Kieckhefer and Olson 1974; Ignoffo et al. 1977; Obrycki and Kring 1998). Flightless morphs have been shown to exhibit longer residence times, and sometimes to yield better control of aphids compared to their conspecifics capable of flight (Lommen et al. 2008; Seko et al. 2008). The major obstacle for the commercialization of wingless *A. bipunctata*, however, is their reduced fitness (Ueno et al. 2004) which complicates mass breeding. Earlier work indicated that the extent of wing reduction in wingless *A. bipunctata* is negatively correlated to components of fitness (Ueno et al. 2004). Our artificial selection experiment corroborates this, because the number of offspring produced by the lines selected in the upward direction is higher than that in the corresponding line selected downward. The ability to artificially select for less wing reduction, in combination with a specific rearing temperature, as shown in this paper, could thus provide a very practical way of improving fitness. This method could ease commercial-scale breeding and, provided their flightless behaviour is retained, may also enhance the wingless ladybird's effectiveness as a biological control agent. This would provide an example of how evolutionary genetic principles can be exploited to improve pest management (Gould 2008).

Acknowledgements

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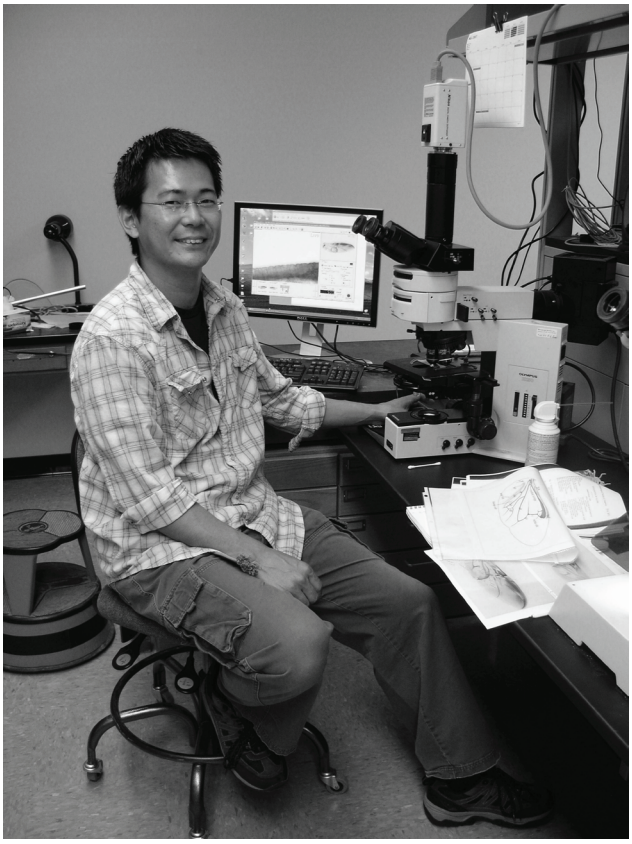

Suzanne Saenko and Yoshi Tomoyasu studying wings

6

Development of a wingless morph in the ladybird beetle, *Adalia bipunctata*

Suzanne T. E. Lommen, Suzanne V. Saenko, Yoshinori Tomoyasu, and Paul M. Brakefield

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Abstract

Many taxa of winged insects have independently lost the ability to fly and often possess reduced wings. Species exhibiting natural variation in wing morphology provide opportunities to investigate the genetics and developmental processes underlying the evolution of alternative wing morphs. Though many wing dimorphic species of beetles are known, the underlying mechanisms of variation are not well understood in this insect order. Here, we examine wing development of wildtype and natural wingless morphs of the two-spot ladybird beetle, *Adalia bipunctata*. We show that both pairs of wings are distally truncated in the wingless adults. A laboratory population of the wingless morph displays heritable variation in the degree of wing truncation, reflecting reduced growth of the larval wing discs. The coexistence of variable wingless morphs supports the idea that typical monomorphic wingless insects may be the result of a gradual evolution of wing loss. Gene expression patterns in wing discs suggest that the conserved gene network controlling wing development in wildtype *Adalia* is disrupted in the dorsoventral patterning pathway in the wingless morphs. Previous research on several species of ant has revealed that the anteroposterior wing patterning pathway is disrupted in wingless workers. Future investigations should confirm whether interruptions in both taxa are limited to the patterning pathways found thus far, or whether there are also shared interruption points. Nevertheless, our results highlight that diverse mechanisms of development are likely to underlie the evolution of wingless insects.

Keywords

Asynchronic development, histology, immunohistochemistry, morphometrics, wing patterning

Introduction

The evolution of flight is an important innovation which has contributed to the success of insects (Wagner and Liebherr 1992). Nevertheless, secondary loss of flight ability has occurred independently and repeatedly in most winged insect orders (Roff 1990) and is often accompanied by loss of wing tissue. In some cases variability in wing morphs is found within species, with examples of both genetic polymorphisms and environmentally induced polyphenisms (e.g. Braendle et al. 2006). Such species provide opportunities to investigate the genetics and the developmental processes underlying the evolution of alternative wing morphs. To date, ants and aphids have been studied in this light. Abouheif and Wray (2002) found that the conserved gene network controlling wing development in winged reproductives of various species of ants was disrupted at different points in the anteroposterior (AP) wing patterning pathway both across and within species to produce wingless worker castes. A genome-wide study of gene expression in nymphs of the pea aphid reported that hundreds of genes, mostly related to energy production, were differentially expressed between winged and wingless morphs (Brisson et al. 2007). Interestingly, about 75% of these genes were only differentially expressed in either polyphenic females or in genetically polymorphic males. In other words, one in four genes showed differential expression in both sexes. Thus, the polyphenism and the polymorphism share some, but not all, gene products with respect to the development of the wingless morph.

The order of beetles (Coleoptera) is a group of insects providing opportunities to extend this knowledge. Flight has been lost in more than thousand species (Roff 1994a) of which some exhibit wing dimorphism (e.g. Darlington 1936; Smith 1964; Dybas 1978; Hammond 1985). Although studies on darkling beetles, including the model species *Tribolium castaneum*, have contributed to understanding the development of beetle wings (Hundertmark 1935; Quennedey and Quennedey 1990, 1999; Tomoyasu et al. 2005), the developmental regulation of alternative wing morphs in this group remains largely unexplored. The two-spot ladybird beetle, *Adalia bipunctata*, is suited to address this issue, because wingless phenotypes occur occasionally in the wild (Majerus and Kearns 1989; Marples et al. 1993). Both the elytra (fore wings) and hind wings are reduced, but other body parts show no apparent morphological changes (Fig. 1B-D, bottom). Earlier work found that this trait is under the genetic control of a recessive allele at a single major locus (Marples et al. 1993). We have collected two beetles with this phenotype, and an individual heterozygote for the trait, from a single locality in three different years. It is highly unlikely that the recessive alleles they carried each represented a different recurrent mutation in the wingless gene, rather a single recessive allele for winglessness appears to be maintained at a low frequency in at least some wild populations. Its study could then help in understanding the evolution of winglessness or wing dimorphism in beetles.

Laboratory populations of the wingless morph in *A. bipunctata* show continuous variation in wing reduction, ranging from individuals exhibiting complete winglessness through to others which lack only small pieces of wing tissue. Nevertheless, we will consistently use "wingless" to describe this genotype. The variation in the degree of winglessness is affected by the genetic background (Ueno et al. 2004) and by environmental factors (Lommen et al. 2005).

In this paper, we examine the developmental processes underlying genetic wing dimorphism in *A. bipunctata* and genetic variation of wing reduction within the wingless morph. Wings can be reduced in a variety of ways, such as miniaturization, deletion of different parts of the wing, or a combination of both. Since the nature of reduction could provide candidate developmental mechanisms, we have first used comparative morphological studies to characterize the size and shape of the reduced wings in wingless adults. Because beetle wings begin to develop in the larva, we have then examined this process in wildtype and wingless phenotypes histologically. Finally, we have chosen three wing development genes known from *Tribolium* and other insects, and examined their expression patterns in both phenotypes.

Materials and methods

Insects

A laboratory stock of wingless two-spot ladybird beetles (*A. bipunctata*) was established by outcrossing two individuals (one a wingless homozygote and the other a heterozygote) found in Utrecht, The Netherlands, with more than a hundred wildtype beetles from the same population. We also started a pure wildtype laboratory stock from the Utrecht location (only for histological examinations of wildtypes, we used a laboratory stock originating from Koppert B.V.). All beetles were maintained in a climate cabinet at a constant temperature of 20.5ºC ± 1.5ºC (RH 65% ± 5%) and a photoperiod of 16L:8D. Both larvae and adults were fed with *Ephestia kuehniella* Zeller eggs with adults receiving a supplement of flower pollen.

Morphology of pupal and adult wings

We used wing colour patterns and wing structures as landmarks to compare wingless beetles with winged ones. To obtain landmarks on the elytra we crossed a winged mutant with a spotty colour pattern spanning the entire elytra (Majerus and Kearns 1989) (Fig. 1E) into the wingless stock. In this way, we reared over a 100 spotty wingless adults. Veins, hairs, sensory organs and pigmentation patterns served as landmarks in hind wings. To examine the size of reduced wings, we took morphometric measurements from 99 wingless adults (37 males, 62 females) and compared them to 73 wildtypes (35 males, 38 females). The wingless adults were chosen such that the range

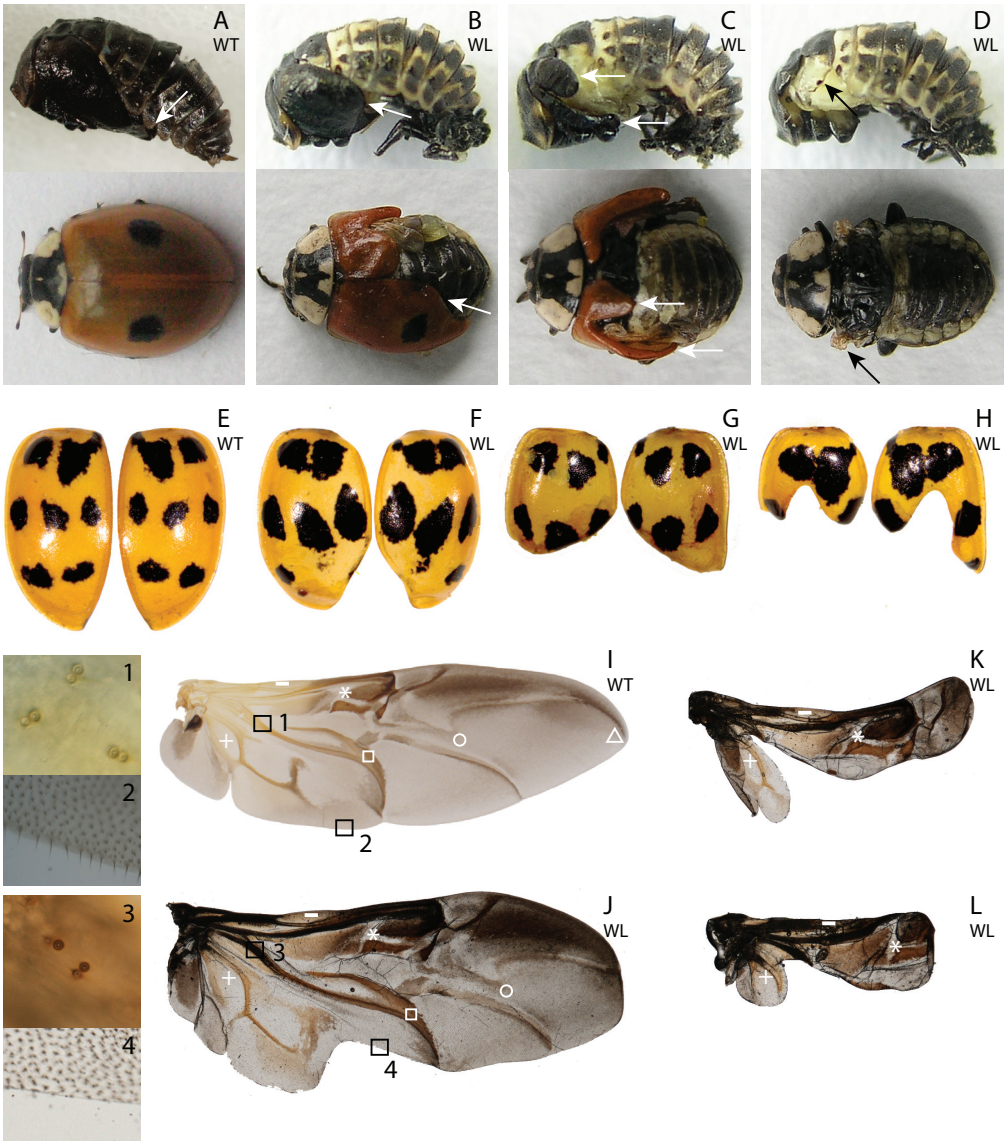


Figure 1. Wings of wildtype (WT) and wingless (WL) beetles demonstrate wing variation in *Adalia bipunctata*. A. WT pupa (top) and adult (bottom). Black drop-like structure in pupa is the wing case, with arrow indicating the distal tip. **B-D.** The size and shape of the pupal wing cases of WL beetles (white arrows in top pictures) correspond to that of elytra in the adults emerging from it (white arrows in bottom pictures). Pupa and adult in D. have no elytra tissue, but hind wings are visible (black arrows). **E-H.** Elytra of individuals homozygote for the *spotty* colour pattern allele indicate that elytra of WL beetles (F-H) are truncated rather than miniaturized, compared to beetles with wildtype wing development (E). **I-L.** The venation pattern and the pigmentation pattern on hind wings of WL beetles show that hind wings are also truncated rather than miniaturized. **I.** WT hind wing with 1. sensory organs on the Medial vein, and 2. long hairs at the posterior wing margin. Symbols represent some of the landmarks, either venation or pigmentation patterns. **J-L.** Hind wings of WL beetles. 3. shows that sensory organs on the Medial vein are similar as in WT. 4. shows that the posterior margin is truncated, since the black hairs typical of that margin in WT are absent. **B, C, H, and J-L** show wings with a typical "lobed" shape, with the wing consisting of two lobes.

of variation was captured in the sample. All insects were killed by freezing at –80^{0}C. On the wildtype adults the maximum length of the left elytron, and for wingless beetles the maximum length of the medial and outer part of the left elytron, were measured (Fig. 2A). Following that, the left hind wing was detached and mounted on a slide, and its maximum length was measured.
To better understand possible changes in the wing at the pupal-adult moult, we visually inspected the pupal wing cases (the cuticle covering the pupal wings, Fig. 1A) of 35 wingless and 35 wildtype beetles and compared them to the shape of the elytra in the adults after they had eclosed.

Histological examination of wing disc growth

We histologically examined the epidermis of the meso- and metathorax which contain the primordia (or wing discs) of elytra and hind wings, respectively (Fig. 3A), in larvae of wingless and wildtype phenotypes. Since we were also interested in variation within the wingless phenotype, we established three pure wingless families from parents lacking the major part of wing and elytron tissue. We used a scale from 0 (complete lack of elytra) to V (elytra approaching wildtype phenotype) to quantify their degree of winglessness as described by Lommen et al. (2005). All parents were scored as class 0, I or II (Table 1). About 60 larvae per family were used for histological studies and the remaining siblings were reared through to adulthood, and then scored for degree of winglessness. The same number of larvae from the Koppert wildtype stock was used for histological study.
Adalia bipunctata has four larval instars and pilot studies with wildtypes showed that wing disc growth does not begin until the third larval instar (L3). Therefore, we used larvae in stage L3, early-L4 (within 1 day after moulting), mid-L4 (1-2 days after moulting), and late-L4 (3-5 days after moulting, a few individuals were already a prepupa). Each wingless family and the wildtype stock contributed 7-29 larvae to every larval stage used. Larvae were anaesthetized with chloroform, and meso- and metathoraces were then fixed in 4%-paraformaldehyde in phosphate buffer (1x PBS, pH=7.2) for 2h. After dehydration in ethanol and Histo-Clear (Agar Scientific), the samples were embedded in paraffin, cut into transverse sections of 8-9 µm (Fig. 3A), stained with Mayer's haematoxylin and eosin, and photographed.

Gene expression patterns in wing discs

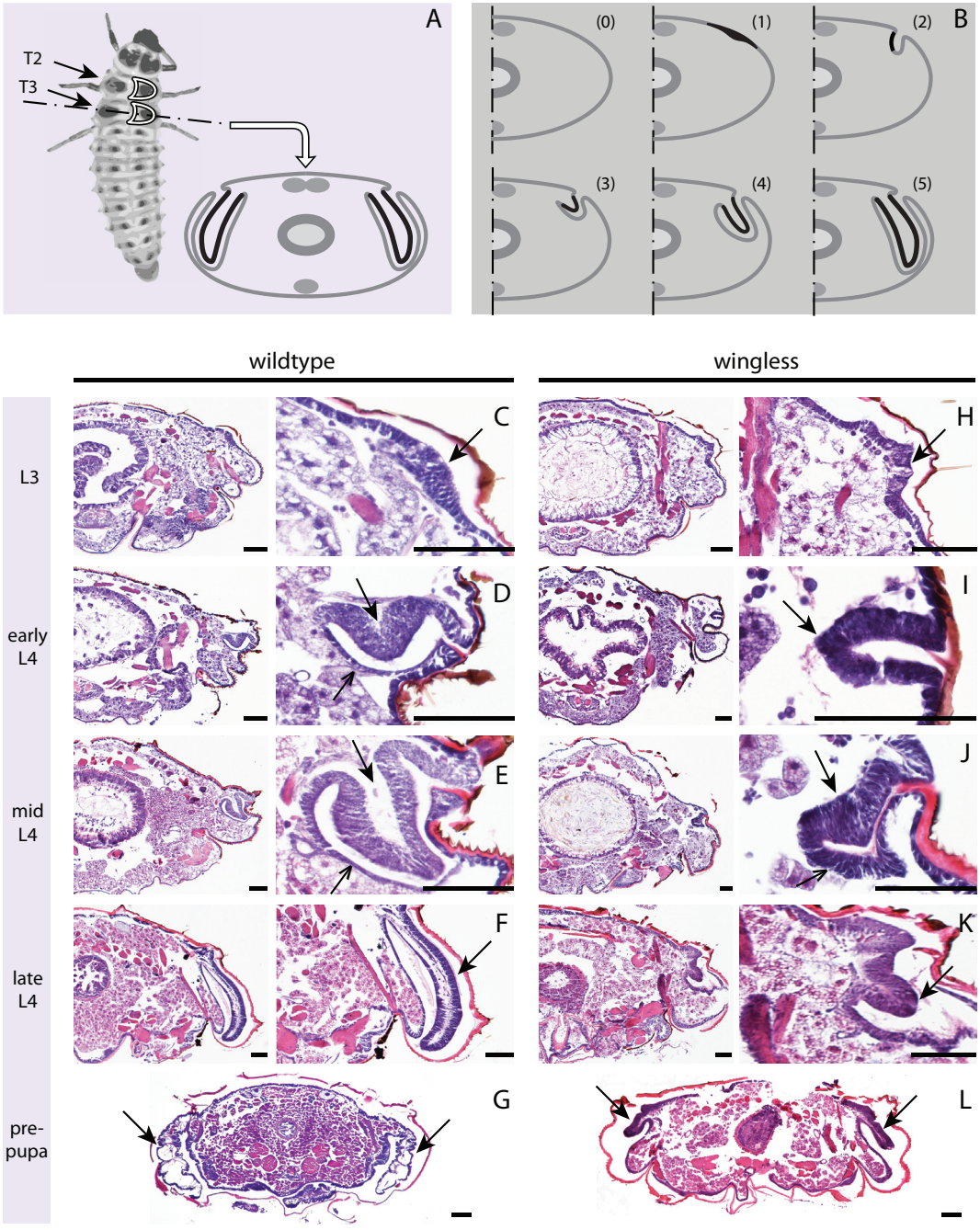
We investigated the expression patterns of *Distal-less* (*Dll*), *engrailed/invected* (*en*), and *pdm/nubbin* (*nub*) in larval wing discs, using antibodies against their protein products. We selected larvae from mid-L4 and late-L4 stages from the wildtype and wingless stock, since younger larvae yield insufficient tissue for dissection. They were anaesthetized with chloroform for 15 minutes and the thorax was then fixed for 30 min in 0.1 M PIPES (pH 6.9), 1 mM EGTA, 1% Triton x-100, 2 mM $MgSO_4$ and 9% formaldehyde. After excision of parts of the epidermis containing a wing disc,

the protocol of Brunetti et al. (2001) was followed for antibody staining. Samples of elytra and hind wings were kept separately in order to compare their gene expression patterns, and samples from the left and right side received antibodies against different gene products. We used a polyclonal antibody against Dll (1:100) (Panganiban et al. 1995), the monoclonal antibody 4F11 against En/Inv (1:100/1:200) (Patel et al. 1989), and a monoclonal antibody against Pdm/Nub (1:10/1:20) (Damen et al. 2002), and used Alexa Fluor 488 (1:200) and Texas Red (1:200) (Molecular Probes) as secondary antibodies. The samples were fixed for 30 min in 9% formaldehyde in wash buffer (cf. Brunetti) and were washed before the wing discs were separated from the epidermis and mounted in pure glycerol. Sample sizes were 5-20 for each combination of phenotype, wing type, developmental stage, and gene product. Exceptions were mid-L4 larvae from wingless beetles, from which we obtained only a few samples due to their minute size, and Nub, which was not tested in any mid-L4 larva. We also included unstained controls to check autofluorescence, and controls that only received secondary antibodies to test for non-specific binding. Images were obtained by confocal laser-scanning microscopy.

Statistical analysis

Measurements on adult wing sizes were analysed using the R statistical package (version 2.6.1., R Development Core Team 2007). Since the data of wingless beetles were not normally distributed and variances were unequal among groups, we applied non-parametric statistics or generalised models. We used a Spearman Rank Correlation test to reveal the relationship between maximum elytron and hind wing length for each sex in each wing phenotype. To compare this relationship between the wing phenotypes we then fitted regression lines to the data, using a Linear Model with Generalized Least Squares, fitted by Restricted Maximum Likelihood, to account for unequal variances. Maximum hind wing length was used as the response variable, while maximum elytron length, wing phenotype and sex were used as explanatory variables. This maximum model was then simplified by stepwise removal of non-significant factors, as revealed by Chi-square tests on the deviances, calculated by the log likelihoods, of the original and the simplified models. We used a Wilcoxon Signed Rank test to examine differences between lengths of the medial and outer parts of elytra of wingless beetles.

Histological data were analysed in SPSS 15.0. Using the histological transverse sections of wildtype larvae, we identified a set of successive morphological changes characterising wing development (see results and Fig. 3B). We then used this set to quantify the variation in wing disc growth of wingless beetles by scoring the development of the largest wing disc in each pair of wings in the transverse sections of each specimen. The largest elytron and hind wing disc differed in score in only 17% of the individuals, and not in any consistent pattern. Therefore, we only used the highest score of each specimen as an ordinal numeric variable for non-parametric analysis of wing disc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growth. For each larval stage dissected, we first tested by a Kruskal-Wallis test whether the three wingless families could be pooled. This was possible for stages L3 and early-L4, and the pooled data were then compared to the wildtype group by a Mann-Whitney U test. Because families could not be pooled for stages mid-L4 and late-L4, we used a Kruskal-Wallis test to examine overall differences among the three wingless families and the wildtype group, followed by multiple pair wise Mann-Whitney U tests with a Bonferroni correction. The same procedure was followed to test for differences among the three wingless families in the frequency distribution of the degree of winglessness of siblings reared to adulthood.

Results

Morphology of pupal and adult wings

Though size and shape of wings of wingless *A. bipunctata* are extremely variable, there are clear patterns in the mode of reduction. Both in the elytra and in the hind wings, morphological landmarks show that the wings are reduced by distal truncation, and in addition, a posterior part of the wings, variable in size and shape, is missing (elytra in Fig. 1F-H, compared to wildtype in E; hind wings J-L, compared to wildtype in I). Rarely, the anterior part of the wing is not well developed. Apart from deformations in size and shape, and sometimes in structure, the truncated wing of wingless beetles resembles the corresponding part in the wildtype wing. Thus, pigmentation patterns, veins, sensory organs, and hairs are present on truncated hind wings at the correct position (elytra in Fig. 1F-H, compared to wildtype in E; hind wings J-L, compared to wildtype in I).

Figure 3. Development of wing discs in larvae of wildtype (WT) and wingless (WL) *A. bipunctata*. **A.** The second (T2) and third thoracic segment (T3) of the larvae bear pigmented spots (black arrows), below which the wing discs develop (white outlines at the larva's right lateral). Larvae were cut transversely (dashed line) to obtain sections (cartoon at the right). The outline symbolises the epidermis, the inner circle symbolises the larval digestive track, and the wing discs are laterally in black. **B.** Cartoons showing subsequent characteristic phases of wing development, numbered 1-5. Drawings represent the right side of transverse cross sections through the larval meso- or metathorax. Wing disc growth starts with thickening of the epidermis (phase 1), followed by a first (phase 2) and second (phase 3) invagination that forms the wing which subsequently expands ventrally (phase 4) until it is more than 4x its width (phase 5). **C-L.** Transverse cross sections of wing discs in WT (overviews of half the body in left panels of **C-F**, with close-ups in the right panels, complete section in **G**) and WL phenotypes (overviews of half the body in left panels of **H-K**, with close-ups in the right panels, complete section in **L**) from larval stage L3 to the prepupal stage. Closed arrows point to the wings, open arrows to the wing sacs and bars correspond to 0.1 mm. **C, H.** In L3 epidermal thickening takes place in both phenotypes (phase 1). **D.** After the first invagination of the tissue has taken place (phase 2), a second invagination forms the wing disc and the remaining invaginated cells form the wing sac (phase 3) in early-L4 in WT. **I.** In early-L4, the WL morph has only undergone the first invagination (phase 2). **E.** The wing discs of mid-L4 WT larvae have increased in length (phase 4). **J.** Wing discs of mid-L4 WL larvae have just formed (phase 3). **F.** Late in L4, the wing sac has broken and the wing disc of WT larvae has expanded further (phase 5). **K.** In WL morphs late in L4, the size of wing disc is smaller than that of WT. **G.** In WT prepupae, the wing disc has folded and covers the lateral side of the body. **L.** In WL prepupae, the wing discs have a typical reduced shape and left and right side differ in size.

The length of the truncated elytra and hind wings is highly correlated (r_s=0.700 for males; r_s=0.908 for females; both p<0.001) (Fig 2B). However, comparing the maximum lengths of elytra and hind wings in wingless beetles to those of wildtypes, shows that hind wings are more reduced than elytra (Chi-square, p<0.001) (Fig. 2B). In addition, both the elytra and flight wings of many wingless beetles have a distinct "lobed" shape, consisting of two lobes (Fig. 1B, C, H, J-L, 2A). This has never been observed in adult wildtype beetles. In each sex, the outer lobe is typically larger than the medial lobe (males: median outer and medial lobe are 1.60mm and 0.70mm, respectively; females: 1.88 mm and 0.97 mm; Wilcoxon Signed Rank test, both p<0.001) (Fig. 2C).

The size of the wing cases was reduced to a variable extent in pupae of wingless beetles (Fig. 1B-D) compared to those of winged ones (Fig. 1A). The degree of reduction displayed in each pupa and the shape of the case matched that of elytra in the eclosing wingless adult (Fig. 1B-D, compare top with bottom), suggesting that wing reduction took place before pupation, that is, in the larval stage.

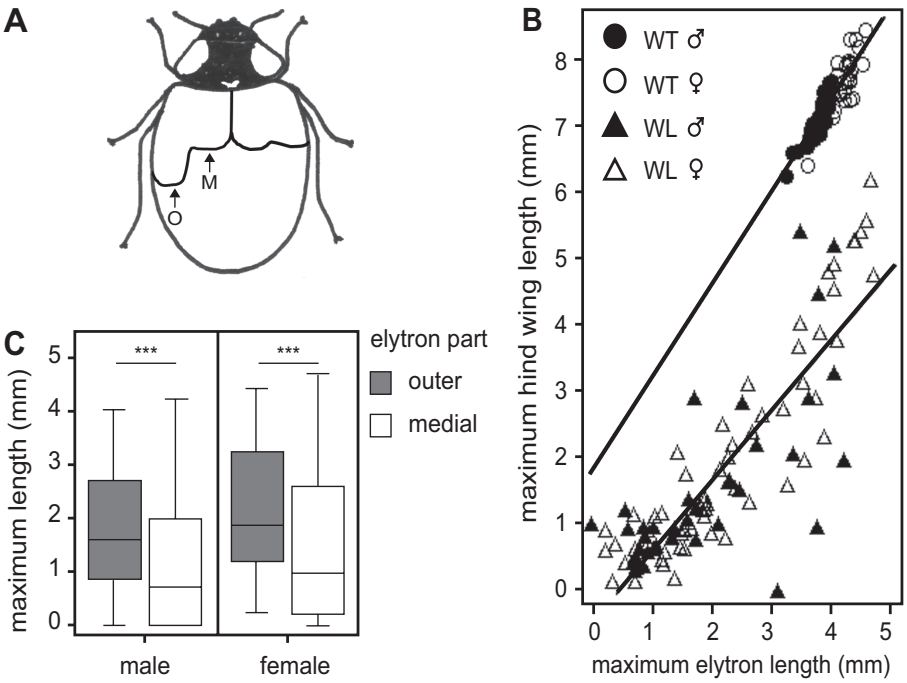


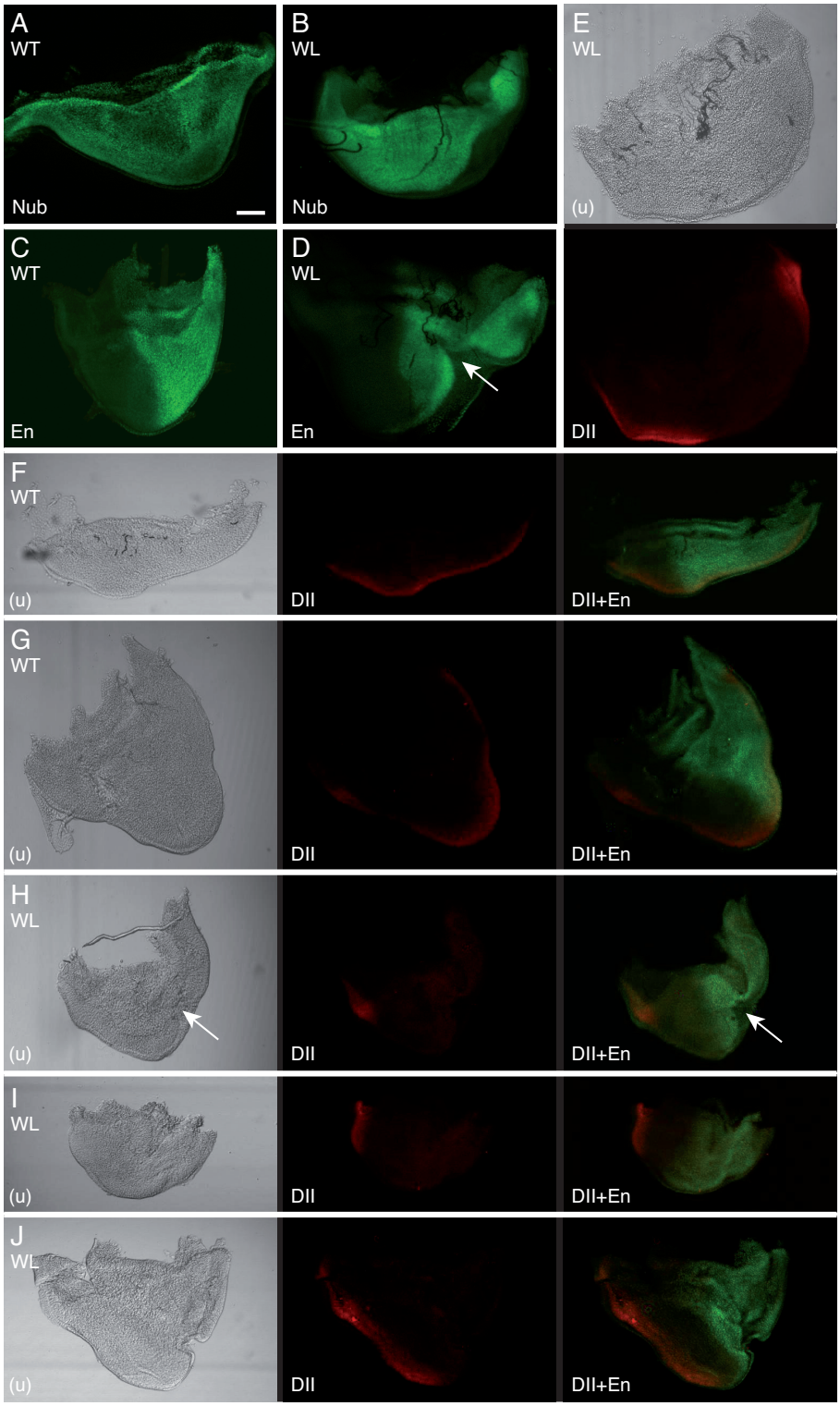
Figure 2. Wing length in *A. bipunctata*. A. The arrows point to the outer (O) and medial (M) part of the left elytron of a wingless individual. **B.** Regression of maximum hind wing on elytron length (mm) of wildtype (WT; y=1.489±0.344 + 1.467±0.099x) and wingless (WL; y=-0.416±0.154 + 1.065±0.060x) male and female adults of *A. bipunctata* shows that WL beetles have reduced wing lengths. The sexes did not significantly differ and showed no interaction with any other factor. In both phenotypes the lengths of elytra and hind wings are correlated, but in WL phenotypes hind wings are relatively more reduced than elytra. **C.** Maximum length of medial and outer elytron parts in wingless beetles. In both sexes, the outer part is significantly longer than the medial.

Histological examination of wing disc growth

Wing disc development in larvae of wildtype *A. bipunctata* corresponds to the description of wing disc development of ladybird beetles by Tower (1903). There is no difference between elytra and hind wing discs until the prepupal stage. Their development begins in L3 by thickening of the epidermal cells of the lateral meso- and metathorax (phase 1 in Fig. 3B; Fig. 3C). In late L3, the cells detach from the cuticle (apolysis). Invagination of the wing disc then takes place either just before or after moulting into L4 (phase 2). In the early-L4, the dorsal layer of the invaginated wing disc thickens and evaginates again to form the future wing (phase 3; Fig. 3D). The thinner ventral layer becomes the wing sac surrounding the wing disc. During the remainder of L4, the wing discs progressively extend ventrally (phase 4; Fig. 3E). The timing of these events varies among individuals by up to several days, but wing discs always increase rapidly in size during late-L4. In 15 of 16 winged individuals, wing length at this stage was over four times the width (phase 5; Fig. 3F). The wing sac then breaks open and the wing surface becomes folded (Fig. 3G). The elytra and hind wings differ in morphology at this stage as the elytron is characterized by a thickened dorsal cuticle.

In larvae of wingless beetles, wing disc growth similarly began in L3, but the remainder of the process was delayed (Fig. 3H-K). As a result, these beetles never had fully formed wings at the prepupal stage (Fig. 3L) but showed a variable degree of development. In none of the stages examined did wingless beetles contain features of apoptosis, such as haemocytes, picnotic cells, and empty spaces between cells.

Figure 4A and 4B show the variation in wing disc development for three wingless families, compared to the wildtype stock. All three wingless families lagged behind in their wing development throughout all stages of development, and the frequency distribution of larvae over developmental phases suggests that the delay in growth increased with time. The results also show differences in wing disc growth within the wingless phenotype. The differences in development between the three wingless families were not significant at earlier stages, but were by the mid- and late-L4 stage (mid-L4: χ_2^2=10.227, p<0.01; late-L4: χ_2^2=18.631, p<0.001). In the late-L4 stage, family A had more fully developed wings than the other families (compared to B: MWU=33.5, p<0.01; C: MWU=29.0, p<0.001), a pattern corresponding to the adult phenotypes (Table 1). Thus, family A, with the smallest lag in wing disc development had parents with the largest elytra, and adult offspring with the longest elytra of any wingless family (compared to B: MWU=416.5, p<0.01; C: MWU=11.5, p<0.001). In addition, adults of family B had longer elytra than family C (MWU=189.5, p<0.01), but this was not reflected in a significant difference in wing disc development in the histological samples of late-L4 larvae (MWU=80.5, p=0.545).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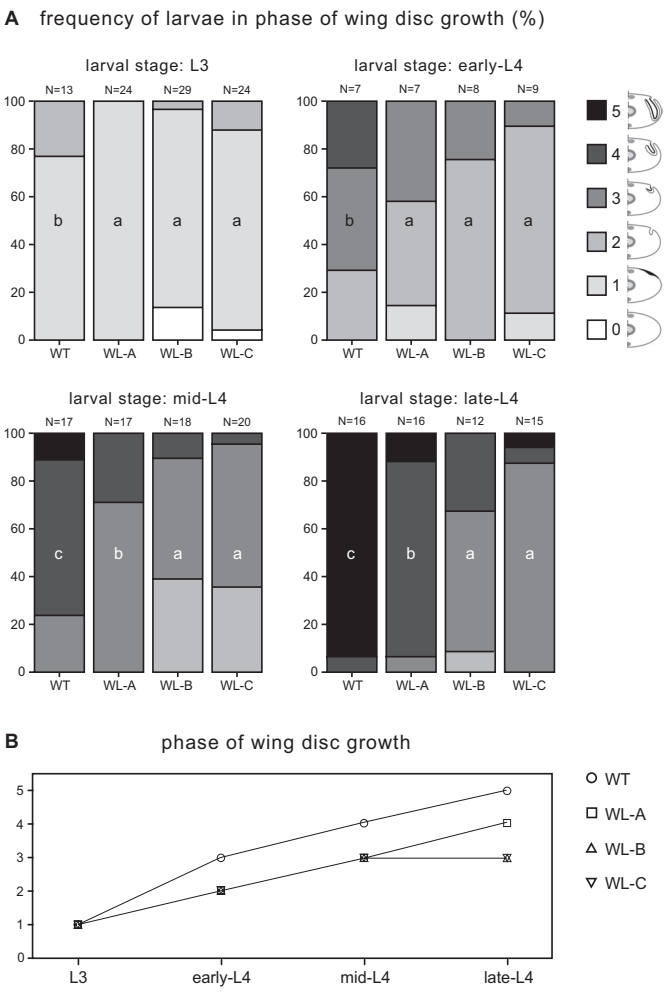

Figure 4. Variation in growth of wing discs in larvae of the wildtype *A. bipunctata* stock and three wingless families.
A. The frequency distribution of the phases of wing disc growth for four larval stages (L3, early-L4, mid-L4, and late-L4). Bars represent the wildtype stock (WT) and the three wingless families (WL-A to WL-C). Stacks represent the percentage of larvae in a particular phase of wing disc growth, the total number of larvae is shown above the upper stack. For each larval stage, significantly different distributions are indicated by different letters ($p<0.05$).
B. Summary of A with symbols representing the median phase of wing development for each group of ladybirds per larval stage.

Figure 5. Gene expression patterns in larval wing discs of wildtype (WT) and wingless (WL) *A. bipunctata* (see next page for Figure). Discs are displayed with the proximal side at the top and the distal tip at the bottom. If En was expressed, the anterior side is displayed at the left, and the (presumed) posterior side at the right. The scale is similar across images (scale bar in A. corresponds to 100µm). **A.** Expression of Nub (in green) throughout the wing tissue in a late-L4 WT. **B.** Similar Nub expression in a late-L4 WL. **C.** Expression of En (in green) in the posterior compartment in a late-L4 WT. **D.** Similar En expression in a late-L4 WL. This expression pattern demonstrates that the two lobes of the wing disc, as indicated by an arrow pointing at the notch separating them, do not correspond to the anterior and posterior compartment. **E.** Wing disc of a late-L4 WL unstained (u) (top), and with Dll expression (bottom, in red). Dll expression is missing distally, compared to WT discs. **F-J.** Panels each show the same wing disc unstained (u) (left), only with Dll expression (middle, in red), and with a double-staining of Dll and En (right, Dll in red, En in green). **F.** Disc of mid-L4 WT with Dll expressed along the entire wing disc margin. **G.** Similar Dll expression in late-L4 WT. **H-J.** Late-L4 WL larvae show that Dll expression is restricted to (part of) the anterior margin. **H.** Dll is restricted to a spot, located proximal anterior. The arrow indicates the separation between the two lobes, which do not correspond to the anterior and posterior compartment as revealed by En expression. **I.** Dll expression is somewhat extended, but still restricted to the proximal half of the anterior margin. **J.** Dll expression covers the anterior margin, but not the posterior margin.

Table 1. Degree of winglessness, classified from 0 (no elytra) to V (elytra covering more than ¾ of the body), in three wingless families of *A. bipunctata* used for histological sections. Values of the parents are given, together with the frequency distribution for their offspring. Significant differences ($p<0.01$) in those distributions are indicated by different letters in the last column.

family	degree of winglessness class		number of offspring per degree of winglessness class							sign
	father	mother	0	I	II	III	IV	V	total	
WL-A	0	II	2	5	1	6	2	0	16	a
WL-B	I	I	38	37	18	7	0	0	100	b
WL-C	0	I	7	1	0	0	0	0	8	c

Gene expression patterns in wing discs

The patterns of Nub, En, and Dll found in *A. bipunctata* larvae were identical in discs of elytra and hind wings. The transcription factor Nub is involved in specifying the early wing disc and plays a role in cell proliferation in *Drosophila* (Ng et al. 1995), while it occurs throughout the prospective wing. As expected, the same pattern was found in wildtype (Fig. 5A) and wingless ladybird beetles (Fig. 5B).

The transcription factor En plays a role in establishment and maintenance of the AP compartmentalization of the insect wing, and is located throughout the posterior compartment in other species of insects (Cohen 1993; Carroll et al. 1994; Abouheif and Wray 2002; Tomoyasu et al. 2005). It might be involved in development of wingless *A. bipunctata* since their wings are often partly truncated on the posterior side. Expression of *en* is also disrupted in wingless castes of several ant species (Abouheif and Wray 2002; Bowsher et al. 2007). En was found in both wildtype (Fig. 5C) and wingless ladybird phenotypes (Fig. 5D), and is indeed confined to one part (the posterior) of the wing discs. We further used this expression pattern to examine whether the anterior and posterior compartments might correspond to the two wing “lobes” often found in wingless *Adalia* (Fig. 1B,C,H,J-L). The “lobed” shape of larval wing discs occurred predominantly in wingless beetles but, surprisingly, also in some winged beetles. In all lobed discs, En covered the smallest lobe completely, and extended into the larger lobe (N=10, Fig. 5D and 5H, right panel).

Because wingless morphs of *A. bipunctata* lack the distal part of both pairs of wings, including the distal wing margin, we were interested in expression of the gene *Dll*. The transcription factor Dll controls normal cell differentiation of the wing margin in *Drosophila* (Campbell and Tomlinson 1998) and is found in wing margins of butterflies (Carroll et al. 1994; Brakefield et al. 1996). In wildtype beetle larvae, Dll was found along the entire margin of the wing discs in both developmental stages examined (Fig. 5F,G). In contrast, in late-L4 larvae of wingless beetles, *Dll* expression was incomplete (Fig. 5E,H-J), with Dll mostly confined to the proximal half of the anterior margin (Fig. 5H,I). In a few

cases Dll was extended to cover more of the anterior side (Fig. 5J) or showed an additional spot at the proximal side of the posterior margin (Fig. 5E). In the few samples of mid-L4 larvae of wingless beetles, Dll was not detected.

Discussion

Mode of adult wing reduction

We have shown that natural wingless phenotypes of the ladybird beetle *A. bipunctata* have two pairs of wings truncated (Fig. 1), which is unusual in two ways. First, hind wings are usually reduced in wingless beetles, but elytra are not. Elytra may have become consolidated, rigidly locked, or rounded after loss of flight (Smith 1964; Crowson 1981), but reduction has only been described for brachypterous rove beetles (Thayer 1992), in several species of brachypterous *Ptiliidae* (Dybas 1978), and in blister beetles (Crowson 1981). Elytra make up such a substantial percentage of the body weight that not having to produce them, or degrading them after they have been formed, is likely to save energy that could be allocated to fitness-related traits (e.g. Zera and Harshman 2001; Lorenz 2007). However, the loss of the elytron may be rare because it is constrained by its protective functions (Crowson 1981).

Second, the "lobed" wing shape typical of wingless adults has to our knowledge not been described for other wingless insects. However, some ant species have workers possessing vestigial wings asymmetric in size of the anterior and posterior compartment (Abouheif and Wray 2002). Using a modelling approach, Nahmad et al. (2008) found that the posterior compartment was likely to be more reduced because of asymmetrical growth, and suggested this was a consequence of the distribution of the Decapentaplegic morphogen, which is predominantly present in the anterior wing part and is required for cell growth.

Developmental mechanisms underlying reduced wings

Two developmental processes have been identified to regulate alternative wing morphs in insects: heterochrony of development and degeneration of wing tissue through apoptosis. These processes may occur in combination and at various stages of wing morphogenesis. For example, wing disc growth is arrested during larval stages in minor workers of the ant *Pheidole megacephala* (Sameshima et al. 2004), and in wingless females of the moth *Eumeta variegata* (Niitsu 2003). Degeneration occurs in nymphal stages of the pea aphid (Ishikawa et al. 2008), in pupae of major workers of *P. megacephala* (Sameshima et al. 2004), and in wingless females of *Diacamma* ants (Gotoh et al. 2005) and of several moths (Niitsu 2001; Lobbia et al. 2003).

Although wing polymorphism has been extensively documented in beetles (e.g. Darlington 1936;

Smith 1964; Dybas 1978; Hammond 1985), the mechanisms of wing reduction in this order are not well understood. In the few documented cases, wing cell degeneration took place in the pupal stage of wingless morphs (Smith 1964, and references therein). In contrast, we find that that freshly pupated wingless *A. bipunctata* already possess reduced wing cases, and that the extent of this reduction is correlated with the eventual reduction of the adult wings (Fig 1B-D). Therefore, wing reduction is probably the result of processes occurring earlier in development. This is confirmed by our histological examinations of wing disc growth in larvae. Cross sections of larval instars of *A. bipunctata* show that the wing discs of wingless morphs follow a similar pattern of growth to those of wildtype beetles, but with a time lag. At the time of pupation, they have substantially smaller wing discs (Fig. 3). Although hind wings are generally more reduced than the elytra in the adult beetles (Fig. 2B), the two pairs of wing discs show a similar growth schedule. This is perhaps because of processes taking place in the pupa, such as expansion of the wing tissue (Smith 1964). The overall pattern suggests that heterochrony, and, more specifically, a change in growth rate of the wing disc, is involved in the process resulting in wingless beetles. However, this cannot explain the distal truncation of the wing discs typical of the wingless morph of *A. bipunctata*, since there is no indication from any system that the insect wing is patterned in a regular proximal-to-distal sequence. Yet, it is unlikely that apoptosis plays any role, since we could not detect any features of this mechanism. It is possible that distal parts are missing from the fate map in wingless beetles, resulting in smaller, incomplete wing discs.

Variation in wing reduction among ladybird families

Our laboratory population of wingless *A. bipunctata* shows wide variation in the degree of winglessness. We examined this variation by making histological studies on three wingless families. The degree of winglessness typical of each family (Table 1) was generally correlated with the size of the wing discs in the larvae (Fig. 4). Overall, the results suggest that wingless beetles have slower wing disc growth, and that variation in the degree of winglessness is associated with variation in the rate of wing disc growth. The differences found among the wingless families in wing disc growth corroborate that this process has a heritable component (Ueno et al. 2004).

A continuous variation in wing reduction has been observed only in a few previous cases (e.g. Darlington 1936). Natural beetle populations exhibiting variation in wing length typically exhibit dimorphism with little variation in the wingless morph (Darlington 1936; Smith 1964). Monomorphic wingless phenotypes appear to have regularly become fixed in evolution, whereas the cases with continuous variation may reflect transitory phases which sometimes progress to fixation in time (Den Boer et al. 1980). Our results support the idea that complete wing reduction could evolve gradually in *A. bipunctata* if favoured by natural selection. Indeed, artificial selection in the laboratory has effectively led to lines showing near fixation of such an extreme wingless phenotype (this thesis, chapter 5).

Conservation of insect wing development

We explored expression patterns of three genes (*nub*, *en*, and *Dll*) involved in insect wing development in wing discs of winged and wingless *A. bipunctata* (Fig. 5). In *Drosophila*, their products act as transcription factors each in a different major wing patterning pathway: Nub is in the body wall/ wing patterning pathway which differentiates body wall and wing tissues (Ng et al. 1996), En is involved in initiating the AP wing patterning pathway (Held 2002), and Dll acts downstream in the dorsoventral (DV) wing patterning pathway (Held 2002).

The expression patterns in winged *A. bipunctata* were in agreement with those found in other insect taxa, such as flies, butterflies and ants (Cohen 1993; Carroll et al. 1994; Ng et al. 1995 ; Brakefield et al. 1996; Abouheif and Wray 2002), as well as in the beetle *T. castaneum* (Tomoyasu et al. 2005; unpublished results Y. Tomoyasu). These results support the idea that wing development is regulated by highly conserved pathways throughout the holometabolous insects (Abouheif and Wray 2002; *Tribolium* Genome Sequence Consortium 2008).

The DV wing patterning pathway is a candidate pathway for the development of wingless morphs

Expression of *nub* and *en* in wingless ladybird beetles was similar to that in wildtype beetles, and therefore not associated with the wingless phenotype (Fig. 5A-D). *En* expression in the posterior compartment of the wing discs also showed that the two “lobes” often seen in wingless beetles do not correspond to the AP compartmentalization (Fig. 5D, H). In contrast, the expression of *Dll* differed between winged and wingless beetles. From *Drosophila* (Campbell and Tomlinson 1998) and *Tribolium* (unpublished results Y. Tomoyasu), it is known that *Dll* is involved in specifying margin structures. In wildtype *A. bipunctata Dll* is expressed similarly at the wing margin (Fig. 5F-G). In contrast, *Dll* expression in wingless ladybird beetles is restricted to the proximal anterior margin (Fig. E, H-J). Interestingly, this location corresponds to the part of the wings that is typically most fully developed in adult wingless beetles. These results suggest an association between *Dll* expression and the wingless genotype. However, although down-regulation of *Dll* by RNAi in late larval stages reduces wings in the beetle *T. castaneum* (unpublished results Y. Tomoyasu) and the ladybird *Harmonia axyridis* (personal communication T. Niimi), it seems unlikely that *Dll* itself specifies development of the wingless genotype in *A. bipunctata*. First, the truncation of distal parts of the wing tissue can extend well beyond the wing margin where *Dll* is expressed in *A. bipunctata*; in the *Dll*-down-regulated *T. castaneum* beetles, only the wing margin is absent (unpublished results Y. Tomoyasu). Second, beetles with a mutation in *Dll* or down-regulation of *Dll* have truncated legs and antennae (Beermann et al. 2001; Niimi et al. 2005, unpublished results Y. Tomoyasu), whereas this is not found in wingless *A. bipunctata*. Therefore, the observed pattern is more likely to result from changes in the regulation of *Dll*. Alternatively, from *Drosophila* mutants it is known that mutations in many genes in the DV wing patterning pathway can result in truncated

wings, including *apterous, Notch, vestigial, scalloped, Beaded, Beaded-Serrate, Beadex, cut*, and *rudimentary* (overview in Lindsley and Zimm 1992), and combinations of these can cause more severely truncated phenotypes (Jack and DeLotto 1992). An association mapping approach could help to examine those alternatives.

We have observed variation in the disturbed *Dll* expression within the wingless morph of *A. bipunctata* (Fig. 5E, H-J). We hypothesize that this variation is associated with the degree of winglessness in the adult morphs. This should be examined further in the future, for example by using families differing in degree of winglessness. If, in addition, a functional relationship were to be found between the location of Dll and the degree of winglessness in *Adalia*, variation in the location of gene expression may provide a developmental mechanism underlying gradual evolution of wing loss, as discussed previously. In contrast, Nahmad et al. (2008) suggested from a modelling approach that a gradual evolution of wing loss involves a single key regulatory gene suppressing wing development in a dose-dependent manner. It is not known whether the gene *Dll* can act in such a manner.

In the wingless castes of all ant species examined to date for gene expression patterns, the gene network underlying wing development was disrupted in the AP patterning pathway (Abouheif and Wray 2002; Bowsher et al. 2007) which acts largely independently of the DV pathway (Held 2002). Thus, *A. bipunctata* provides the first example of a natural wingless phenotype associated with disruption in the DV patterning pathway. We cannot exclude, however, that *A. bipunctata* and ants also share interruption points until more genes of both patterning pathways have been examined. We have also shown that the phenotype of wingless *A. bipunctata* is unusual among wingless insects. Together, these results suggest that even though the gene network regulating wing development is conserved in winged morphs, different developmental mechanisms can produce winglessness and might have been involved in the evolution of wingless morphs throughout the insect world. It remains to be investigated which changes on the molecular level are controlling the development of wingless morphs, and this should be examined in more species of insect. This will provide opportunities to sort out whether differences in the nature of winglessness, such as polyphenism versus genetic polymorphism, and adaptive phenotypes (as in aphids and ants) versus low frequency mutations segregating in natural populations (as in *A. bipunctata*), mirror differences in developmental processes.

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Ellyn Bitume feeding ladybirds with aphids

7

Touch matters in sex: natural variation in wing length reveals tactile mating cues in a ladybird beetle

Suzanne T.E. Lommen, Ellyn V. Bitume, Peter W. de Jong, and Paul M. Brakefield

Abstract

The elytra of many beetles play a role in mating, by providing olfactory, visual, or audible cues. In the predatory ladybird beetle, *Adalia bipunctata*, cuticular hydrocarbons covering the elytra function as contact sex pheromone. Other potential cues are not well studied in this species. Here, natural variation in the length of the elytra in *A. bipunctata* is utilized to examine their role in mating. In wingless morphs, both pairs of wings are truncated. Wingless individuals are homozygous for a recessive wingless allele, but vary in their extent of wing reduction. We recorded the behaviour of the ladybirds in laboratory mating assays where single males were offered either one or two females. Comparing the behaviour of different genotypes provided no evidence for pleiotropic effects of the wingless locus on the mating behaviour of individuals of either sex in heterozygotes or homozygotes. In contrast, mating was strongly affected by the female phenotype but not that of the male. The incidence of mating declined with female elytra length in no-choice tests, and in choice tests with single winged and wingless females, the latter were never mated. The results strongly suggest that elytra provide tactile mating cues but no visual cues in this species. We further discuss how these tactile cues could work, and then examine the implications of these findings for the evolution of winglessness and for the use of wingless ladybirds as biological control agents.

Keywords

Adalia bipunctata, behavioural observations, cuticular hydrocarbons, choice test, gas chromatography, tactile cue, visual cue, winglessness

Introduction

Cues in beetle mating behaviour

Mating behaviour of beetles (Insecta: Coleoptera) has been widely studied, especially in species of economic importance such as predators and pest species. Males are typically the most active sex in mate-searching and initiating mating behaviour, and a variety of cues for male mating behaviour have been identified. These include olfactory signals such as cuticular hydrocarbons (CHCs, "contact sex pheromones") (e.g. in Curculionidae, Mutis et al. 2009; reviewed for Cerambycidae by Ginzel 2010; e.g. in Lampyridae, Ming and Lewis 2010) and volatile hydrocarbons (reviewed by Bartelt 2010), as well as visual (in Cerambycidae, Fukaya et al. 2005; Lelito et al. 2007; e.g. in Buprestidae, Lelito et al. 2011) tactile (e.g. in Chrysomelidae, Geiselhardt et al. 2009), and female behavioural cues (e.g. in Coccinellidae, Hemptinne et al. 1998). Experimental work is often focussed on a single type of cue, but some studies show that combinations of several cues can be involved in mating behaviour within a particular species, either simultaneously or sequentially (Hemptinne et al. 1998; Fukaya et al. 2004; Fonseca and Zarbin 2009; Yasui 2009; Lelito et al. 2011; Yasui et al. 2012).

Role of elytra in mating

Many studies cover mating cues associated with the elytra, the modified fore wings typical of beetles. Elytra usually cover the flight wings and most of the body, including part of the thorax and the entire abdomen. In many species of beetles, mating occurs with the male positioned on top of the female, thus holding her elytra. Evidently, elytra play an important role in mating, and they can provide multiple cues. For example, CHCs cover the entire body (Howard and Blomquist 1982), but given the large portion of the outer surface that is made up by this structure, and the dorsal mating position of many males, the CHCs on female elytra are the most likely body part to be contacted by a potential mating partner. Indeed, a large body of experimental work concerns male mating behaviour in response to removal of CHCs from female elytra and re-application on to both live and dead females and to artificial models (e.g. Peschke 1978; Jermy and Butt 1991; Fukaya et al. 1996; Hemptinne et al. 1998; Ginzel and Hanks 2003; Luo et al. 2011). It has been shown that CHCs are generally species-specific (e.g. Golden et al. 1992; Peterson et al. 2007; Tanigaki et al. 2007), and may be gender-specific (Ginzel et al. 2003), and condition-dependent (Peschke 1987; Steiger et al. 2007; Steiger et al. 2009). Elytra can also provide visual signals, and the role of these signals has been studied by blinding males (Pureswaran and Poland 2009) and by using rods of different colours and sizes as surrogate females (Fukaya et al. 2004; Fukaya et al. 2005). The tactile cues that elytra can provide are, however, not frequently studied.

***Adalia bipunctata* as a model in this study**

In this study, we tested the hypothesis that elytra can also play a role in mating behaviour by means of tactile cues, using the predatory two-spot ladybird beetle, *Adalia bipunctata* (L.), as a model species. Hemptinne et al. (1996; 1998) have shown that males of this species use CHCs and female-specific behaviour to recognize potential mating partners, but they found no evidence for volatile chemical cues. To investigate the putative tactile cues of the elytra, we exploited intraspecific natural variation in wing length in this species. Wingless individuals occasionally occur in the wild (Majerus and Kearns 1989; Marples et al. 1993). This trait is genetically controlled by a single locus with the wingless allele recessive to the wild-type allele (Marples et al. 1993). Genetically wingless beetles possess truncated elytra and hind wings, and the extent of wing reduction is correlated between the two pairs of wings within individuals (Lommen et al. 2009). However, the expression of the wingless trait is highly variable between individuals (Marples et al. 1993; Lommen et al. 2009). Thus, in a wingless laboratory stock of this species, phenotypes range continuously from those without any wing tissue to those indistinguishable from the wild type. This variation in the degree of wing reduction makes it a uniquely useful model organism to examine the structural function of elytra avoiding the risk of damage associated with trimming down this structure artificially.

We report here on a series of mating behavioural assays comparing winged and wingless *A. bipunctata.* In control experiments we assess potential pleiotropic effects of the wingless allele on mating behaviour or mate choice. We examine the results in light of the function of elytra in mating, and then discuss observed variation in mating behaviour in relation to the evolution of winglessness and the use of this beetle as a natural enemy in biological control programs.

Material and methods

Insect rearing

All *A. bipunctata* used in the experiments came from laboratory stocks. An overview of all genotypes and phenotypes used is given in Table 1. A wingless stock ("WL", -/- for the wingless trait) was established between 2000 and 2004 by outcrossing two individuals bearing the wingless allele to hundreds of winged beetles all collected in Utrecht in The Netherlands (details in Lommen et al. 2005). From this stock a "fully winged wingless" line ("$WL_{F''}$ -/- for the wingless trait), phenotypically close to wild types, was derived by artificially selecting for individuals with minimal wing reductions over several generations. All WL_F beetles used came from this stock. A winged stock consisting of wild types ("WT", +/+ for the wingless trait) was established in 2005 by collecting individuals from the same Dutch locality. For the current experiment we also created a winged stock heterozygote for the wingless trait ("HET", +/- for the wingless trait, note that the wingless allele is recessive) by

Table 1. Genotype, phenotype, description, and origin of all types of beetles tested.

type	genotype	phenotype	description	origin
wingless (WL)	-/-	wingless	variable extent of wing reduction	stock established in 2000-2004
fully-winged wingless (WL_F)	-/-	winged	artificially selected for long wings	derived from the wingless stock
heterozygous (HET)	+/-	winged	wingless gene not expressed	wingless stock crossed with wild-type stock
wild-type (WT)	+/+	winged		stock established in 2005

crossing the WL to the WT stock. All adult beetles were bred in groups separated by wing genotype, sex, and day of emergence from the pupa. They were maintained on a diet of *Ephestia kuehniella* Zeller eggs and flower pollen at a temperature of 20.5 (±1) °C in a 16L:8D light regime.

Mating behaviour

Behavioural experiments were performed from April to August 2007 in a different climate room to that used for rearing. Because elytral colour has been found to affect female mate choice in some populations of *A. bipunctata* (Majerus et al. 1982; Majerus et al. 1986), only *typica* (red) colour morphs were used in the mating behaviour assays. All ladybirds were virgin, and since the incidence of mating is age-dependent, we standardised their age to 21 days at which most females are receptive (Hemptinne et al. 2001). We focused on male behaviour, since they spend most of their time mate-locating (Hemptinne et al. 1996) and mating is usually triggered by a male encountering a female (Hemptinne et al. 1998; S.T.E. Lommen and E.x. Bitume, personal observation). Experiments were performed in small Petri dishes (Ø 55 mm, 14 mm high) to exclude effects of flight ability. A single male was allowed to settle for 15 minutes before one (no-choice experiments) or two females (choice experiments) were added. We then recorded the behaviour of the male for 15 min, as well as the behaviour of the female(s) when interacting with the male. In *A. bipunctata*, mating is preceded by a fixed sequence of male behaviour initiated upon contact between the sexes (Hemptinne et al. 1998). The male first touches the female with his legs (palpate=P), then mounts her, extrudes his genitalia (attempt=A), aligns himself in the right position, and finally penetrates (copulation=C) (Fig. 1). We recorded the occurrence of these events and noted which part of the female was palpated by the male, distinguishing the front (head and pronotum) and rear of the body (remaining thorax and abdomen). We also recorded female rejection behaviour, including running, kicking, raising the abdomen, spinning, and flipping over. Although females can display rejection behaviour in all stages in the chain of sequential behaviours, it did not seem to affect male behaviour, except that it could prevent attempting males to mate or delay mating. Therefore, Figure 1 only displays rejection behaviour upon male attempt, and not earlier in the behavioural cascade.

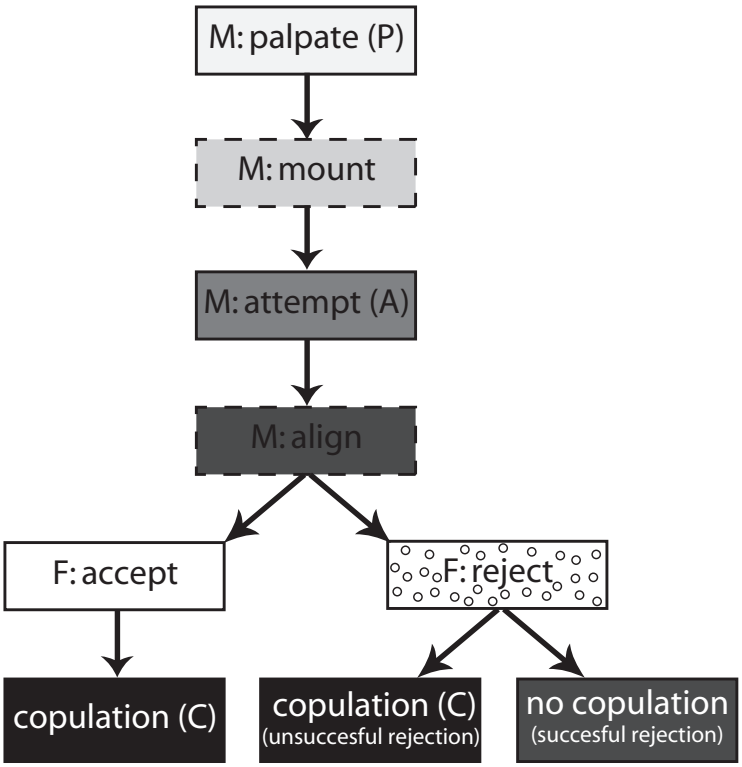


Figure 1. Sequence of mating behaviour in *A. bipunctata*. M=male, F=female behaviour. Letters in brackets indicate abbreviations used throughout the manuscript. Only behaviour in text boxes with line borders was analysed, not behaviour in boxes with dashed lines.

Individual beetles were only used once. After the trial, all ladybirds were frozen at -20°C before morphological features were assessed. Because size can affect chance of mating in *A. bipunctata* (Tomlinson et al. 1995; Yasuda and Dixon 2002; Perry et al. 2009), we measured maximum head width as an indication of body size. We determined the degree of winglessness (DWL) of all wingless beetles following Lommen et al. (2005). The categorical parameter DWL ranges from class 0 (no elytron tissue) to 5 (elytra covering more than ¾ of the abdomen). DWL classes of wingless beetles are indicated by a lower case number, thus WL_0=wingless beetle of DWL class 0, WL_1=wingless beetle of DWL class 1, and so on. Table 2 gives an overview of the behavioural experiments and their setup. To be able to disentangle potential pleiotropic effects of the wingless allele from direct effects, we set up a series of control experiments comparing winged phenotypes with or without wingless alleles. Subsequently we give additional details for each experiment (Table 2).

Table 2. Overview of the behavioural experiments.

experiment	objective	N	male	female
no-choice	test role of female elytra in mating	192	WT	WL, WL_F or WT
choice (1) between wingless and winged sisters	test effect of winglessness on mating behaviour and male preference	34	HET	WL vs. HET
		34	WL	WL vs. HET
choice (2) between winged females with one wingless allele or without	control experiment to test effect of a single wingless allele on mating behaviour and male preference	40	HET	WT vs. HET
		38	WT	WT vs. HET
choice (3) between winged females with two wingless alleles or without	control experiment to test effect of two wingless allele on mating behaviour and male preference	40	WT	WL_F vs. WT

No-choice experiment

To test the role of elytra in mating, single winged males (WT, N=192) were offered single females, either wild types (WT, N=30) or genetically wingless females, varying in their degree of winglessness (WL_0, N=30; WL_2, N=22; WL_3, N=29; WL_4, N=25; WL_5, N=26; WL_F, N=30).

Choice between wingless and winged sisters

We examined the effect of winglessness on male mating behaviour, including male preference and male-female interactions. By offering males a winged and a wingless female simultaneously, we assessed the effect of winglessness in females on male mating behaviour and preference. By comparing trials between winged and wingless males, we aimed to detect putative pleiotropic effects of winglessness on male behaviour. To minimise differences in the genetic background of the winged and wingless beetles, we created families with winged (HET) and wingless (WL) siblings by crossing single winged males (HET) to single wingless females (WL). A winged and a wingless brother were then each offered a pair of winged and wingless females. All four females were sisters, but were not related to the males (Fig. 1). Each family provided at most two brothers and four sisters, and the experiment was replicated 34 times.

Choice between winged females with one wingless allele or without

To identify putative pleiotropic effects of the wingless allele on mating behaviour, we compared behaviour of winged ladybirds heterozygote for the wingless trait (HET) with those wildtype for the trait (WT). We repeated the setup of the previous choice experiment (N=38 for WT males, and N=40 for HET males), but used beetles from the stocks and not from families. To distinguish WT from HET females in a trial, each of them was marked with a black dot on a different side of the pronotum; the location of the mark was alternated between genotypes over trials.

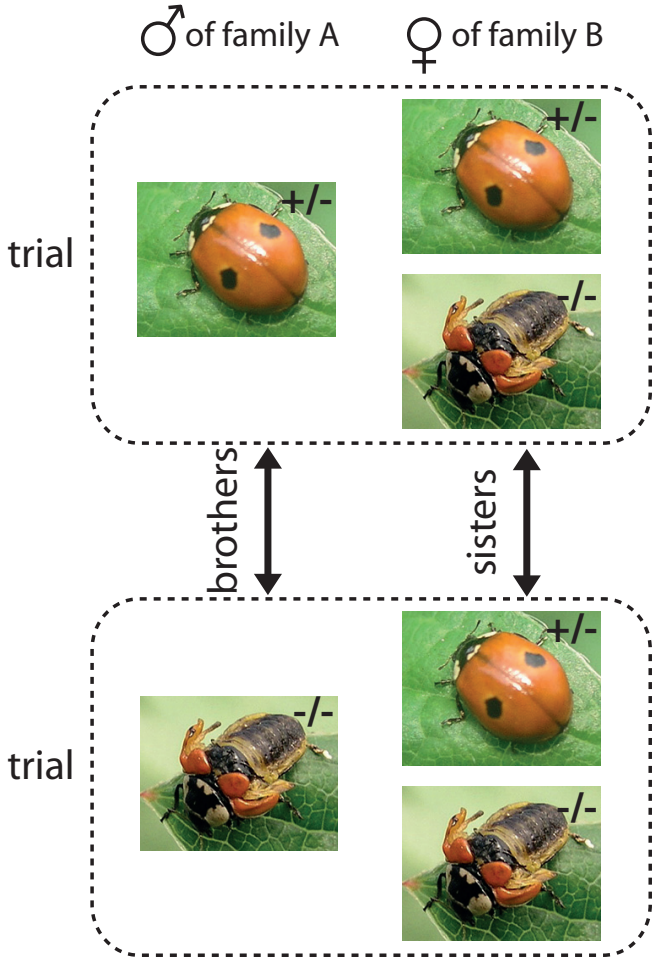


Figure 2. Setup of the mating behaviour experiment examining choice between winged (HET) and wingless siblings. Boxes are separate trials, photos indicate wing phenotypes, and wing genotype is given in the upper right corner of each photo ("+" indicating wild-type allele; "-" wingless allele). Each of the males were full sibs, and each was offered two unrelated female full sibs.

Choice between winged beetles with and without wingless alleles

To test whether female wingless alleles affect male mate choice, we offered single winged males (WT, N=40) a winged (WT) and a fully-winged wingless female (WL_F). Females were thus phenotypically similar, but were genetically different. Pairs of females were marked as above.

Statistical analyses

Data analysis used R software (version 2.6.1., R Development Core Team 2007). The few mating trials where males were so inactive that they never palpated any female were independent of male or female group, and, therefore, excluded from analysis (N=5/192 in no-choice experiment, N=2/68 in choice experiment 1, N=1/78 in choice experiment 2, N=1/40 in choice experiment 3). In

addition, one trial of the no-choice experiment 3 was excluded because the male lacked genitalia. For each experiment, we counted the number of trials in which each of the distinct mating behaviours occurred (for males: palpation, mounting, attempting, copulation; for females: rejection upon male attempt or not, Fig. 1). Since palpated females only occasionally remained unmounted, we did not analyse this behaviour any further. For each trial, we registered how far the male progressed in the chain of sequential mating behaviours (palpation, attempt, or copulation). In choice experiments this was recorded for each of the two females offered. For each female we calculated the amount of time in seconds between each pair of sequential events: time until first palpation (0-P), from first palpation to first attempt (P-A), and from first attempt until first copulation (A-C). In experiments where different male groups were used (choice experiment 1 and 2), we first compared their behaviour and female rejection behaviour towards them. If no significant differences were found, data of all males were pooled for further analysis on female groups.

Generally, Chi-square tests were used to compare frequencies of trials. To examine which factors affected the probability of a specific behaviour to occur in a trial, we fitted generalized linear models (GLMs) with a Binomial distribution and a logit link function. We included a relevant selection of the following factors as explanatory variables: male size, female group, female size, female rejection behaviour, and degree of winglessness. We first fitted fully saturated models and then stepwise removed explanatory variables until the minimal adequate models were found. Variable significance levels were determined from Chi-square tests on changes in the deviance following removal of a variable. In the same way, we applied GLMs with a Gamma distribution and an inverse link function to test whether these factors and (only in the no-choice experiment) the incidence of mating influenced the time lapse between sequential behaviours. However, in this case significance levels are reported from F-tests on the change in deviance to deal with overdispersion (Crawley 2007). For the no-choice tests, we pooled female groups with less than five cases to perform this analysis. In the choice tests, the timing of events was only analysed for the mated female. In choice experiment 1 we only used replicates where both brothers mated.

The relation between body parts and the frequency of these being palpated was only analysed for the no-choice experiment. We compared the frequency of palpation between body parts and female group by Generalized Mixed Effect Models (GLMMs) with a Poisson distribution and a log link function with body part nested within trial as random factor. Significant levels are reported from Chi-square tests on changes in the deviance following removal of a factor (level). Finally, we tested the effect of sex, group, and family (when applicable) on body size by a Student's t-test or an Analysis of Variance (ANOVA).

Results

No-choice experiment

Figure 3 shows how far males progressed in the chain of behaviours towards mating in the no-choice experiments, expressed by the frequency of trials. Although female groups significantly differed in body size (ANOVA, $F_{6,180}$=7.308, p<0.001, with fully winged wingless and wildtype females being smaller than the wingless phenotypes), female body size did not affect the probability of male attempt (GLM, χ_1^2=1.381, p=0.240). Female group, however, significantly affected the probability of an attempt (GLM, χ_1^2=73.949, p<0.001): the chance of a female being attempted significantly increased with the following groups: $WL_0 < WL_2+WL_3 < WL_4 < WL_5+WL_F+WT$. Similarly, copulation did not depend on female body size (GLM, χ_1^2=0.057, p=0.811), but significantly increased with elytron length in the groups $WL_0 < WL_2+WL_3 < WL_4+WL_5 < WL_F+WT$ (Fig. 3). Female rejection occurred in all female groups except WL_0, but never prevented mating (Fig. 3).

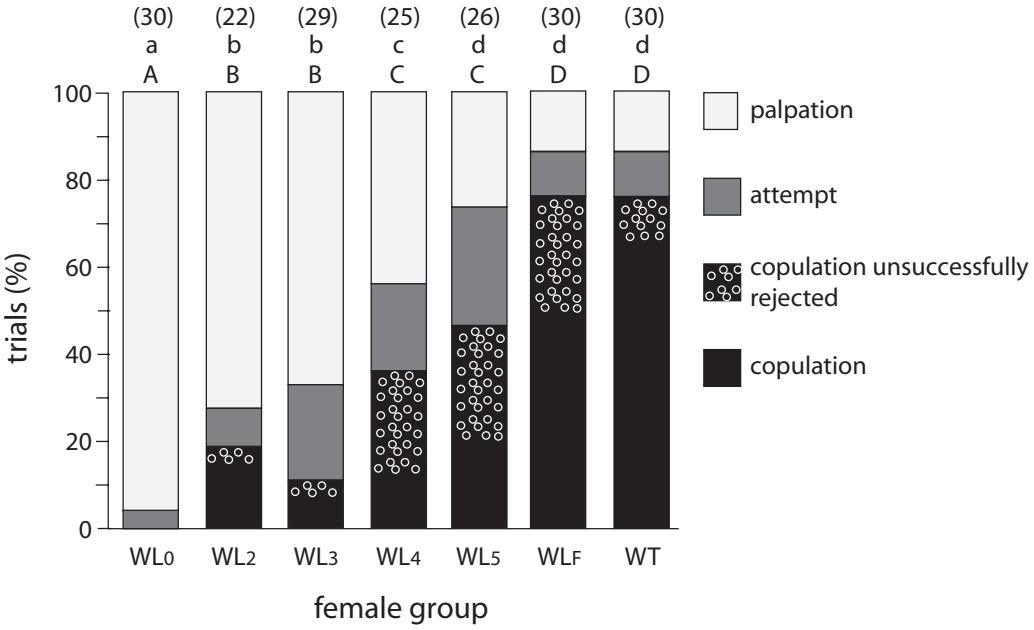


Figure 3. Frequency of trials in which males displayed mating behaviour when offered a single female. Columns represent female groups; six wingless genotypes were used, increasing in elytron length from WL_0 to WL_F (see material and methods), and one group of females wild-type for the wingless trait (WT). Stacks represent behaviour types that males display in fixed sequence from light to dark. Circles indicate female rejection upon male mating attempt. Numbers between brackets represent the number of replicates. Female groups with different letters significantly differ in the number of trials in which a behaviour was displayed (GLM, p<0.05), where lower case letters and capitals stand for the trials with attempts and copulations, respectively.

Further analysis of the frequency of actions within trials revealed that the palpation frequency was higher at the front of the female body than at the rear (GLMM, χ_1^2=25.672, p<0.000). Palpation frequency within trials significantly differed between female groups (GLMM, χ_1^2=42.668, p<0.001), with females bearing longer elytra receiving fewer palpations: WL_F+WT < WL_4+WL_5 < WL_0+WL_2+WL_3. Females phenotypically wingless were more often attempted after palpation on the front of the body than after palpation at the rear (WL_0+WL_2+WL_3: χ_1^2=6.813, p<0.010; WL_4+WL_5: χ_1^2=5.792, p<0.050). For phenotypically winged females, attempts were equally often following palpation at the front, and following palpation at the rear of the body (WL_F+WT: χ_1^2=0.734, p=0.392).

Analysis of the time intervals between consecutive behaviours showed that none of these was affected by female size (GLM; 0-P: $F_{1,179}$=0.769, p=0.382; P-A: $F_{1,91}$=0.268, p=0.606; A-C: $F_{1,64}$=0.000, p=0.999). There was a trend for intervals to shorten with increased in elytron length (Fig. 4A), but differences between groups were never significant (GLM; 0-P: $F_{1,185}$=1.323, p=0.249; P-A: $F_{1,90}$=1.0826, p=0.376; A-C: $F_{1,68}$=1.360, p=0.258). The only factor significantly affecting the time until palpation and time between palpation and attempt was copulation, with shorter intervals for copulated females than those not copulated (GLM; 0-P: $F_{1,186}$=6.335, p<0.050; P-A: $F_{1,92}$=6.495, p<0.050) (Fig. 4A). Female rejection behaviour upon male attempt significantly delayed the timing of copulation (F1,69=11.816, p<0.010) (Fig. 4B).

Summarising, the incidence of mating increased with female elytron length, whereas the palpation frequency per trial decreased.

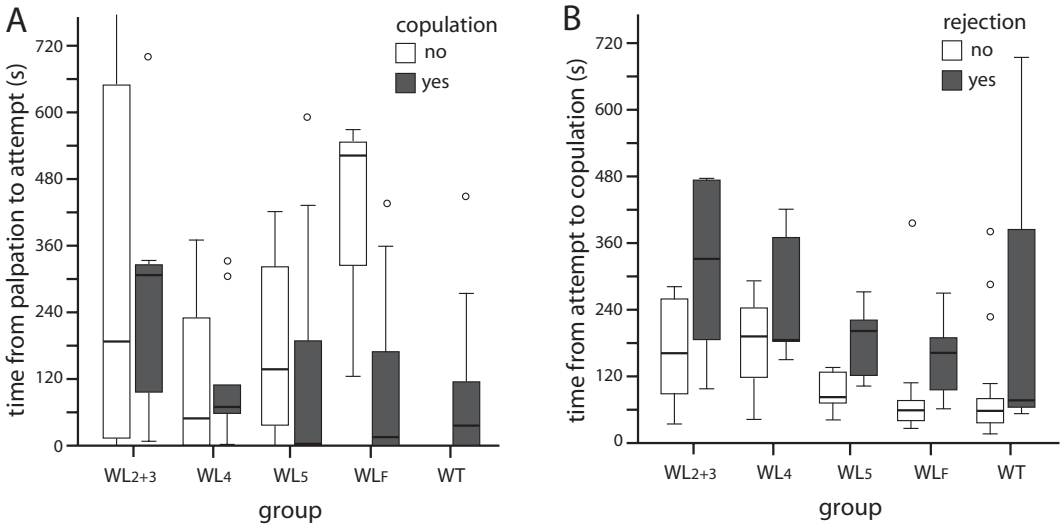


Figure 4. Timing of events preceding copulation per female group. Boxes represent interquartile ranges (IQR), covering 50% of all data points, thick lines in boxes indicate median values, and whiskers show the range of values that fall within 1.5 IQR of the box. Dots represent outliers. A) Time from palpation to attempt is significantly shorter for copulated females (grey boxes) than for females not copulated (white boxes). B) Time between attempt and copulation is significantly prolonged in females that reject (grey boxes) compared to those that do not reject (white boxes).

Choice between wingless and winged sisters

To assess putative pleiotropic effects of the wingless allele on male mating behaviour, we first compared the behaviour of winged (HET) to that of wingless (WL) males. Figure 5 shows the number of trials in which males palpated, attempted and copulated both females, only the winged (HET) female, or none of the females (males never displayed these behaviours exclusively with the wingless (WL) female). These numbers did not significantly differ between the two male phenotypes (all: N=32; palpation: χ_1^2=1.237, p=0.266; attempt: χ_1^2=3.017, p=0.221; copulation: χ_1^2=2.413, p=0.120). Both male phenotypes only copulated with winged females. The number of females that showed rejection behaviour upon male attempt also did not significantly differ between the male phenotypes (N=5 out of 25 attempts by winged males, N=7 out of 29 attempts by wingless males, χ_1^2=0.000, p=1.000), and only rarely prevented mating (N=2 for winged males; N=1 for wingless males).

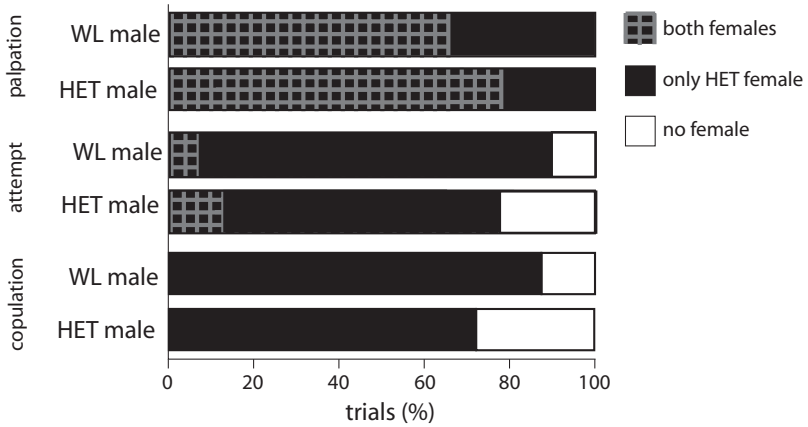


Figure 5. Frequency of trials where either a winged (HET) or wingless (WL) male interacted with winged (HET) and wingless (WL) females when offered both in a choice test (N=32). Winged and wingless males behave similarly, but wingless females are less often palpated, attempted and copulated than their winged sisters.

Winged and wingless males showed similar frequencies of mating behaviour, and the timing of events preceding mating with the winged female was the same. There was no significant difference in the time until they first palpated the winged female (GLM, $F_{1,41}$=3.711, p=0.061), and the time it then took them until their first attempt (GLM, $F_{1,41}$=0.419, p=0.521) (Fig. 6). The time from attempt to copulation was significantly affected by the interaction between male phenotype and female rejection (GLM, $F_{1,39}$=5.853, p=0.020), such that female rejection delayed copulation by wingless males, but not by winged ones. However, this did not result in significant differences between the male phenotypes in mean time from attempt to copulation (GLM, $F_{1,41}$=0.0287, p=0.866) (Fig. 6).

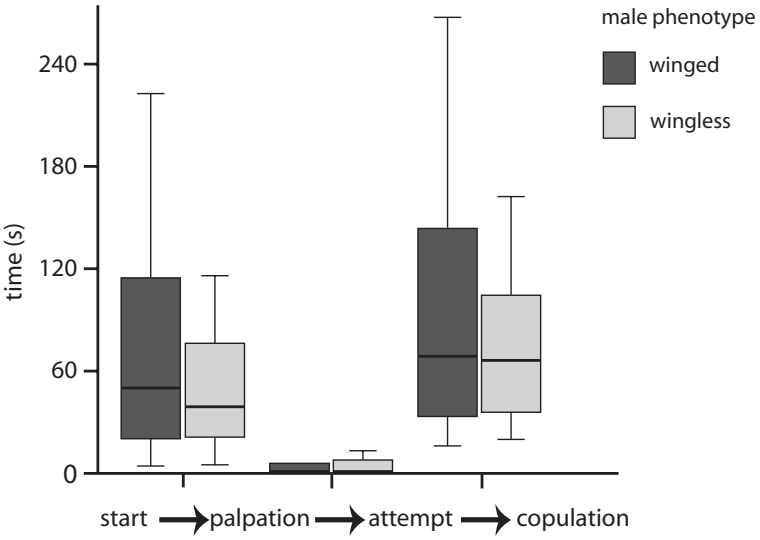


Figure 6. Timing of behaviours by winged (HET, dark bars) and wingless (WL, light bars) males preceding copulation of the winged (HET) female in a choice test with a wingless female (WL, which was never mated) (N=21). Boxplots as in Fig. 3.

The total time from first palpation until copulation was neither affected by male phenotype (GLM, $F_{1,40}$=0.368, p=0.547), nor female rejection (GLM, $F_{1,41}$=01.292, p=0.262), or their interaction (GLM, $F_{1,39}$=3.526, p=0.068).

Since winged and wingless males showed equal patterns in the frequency and timing of behaviours, the male groups were pooled, and we proceeded with the analysis of female effects. The interaction of wing phenotype and sex had a significant effect on body size (ANOVA, $F_{1,195}$=6.847, p<0.01): wingless beetles were wider than their winged brothers or sisters, but the difference was larger for males than for females. Therefore, male and female body size were both included in the analyses. The average degree of winglessness (DWL) class of wingless females was 2.66±1.10 (mean±SD). In most trials both the winged and the wingless female were palpated (Fig. 5), and they were equally likely to be palpated first (χ_1^2=0.563; p=0.453). However, winged females were palpated in more trials than their wingless sisters (χ_1^2=19.920, p<0.000): all winged females, but not all wingless females were palpated. The probability that a wingless female was palpated was affected by her size, with smaller females having a higher chance (GLM, χ_1^2=5.934, p<0.050), but not by her DWL (GLM, χ_1^2=0.696, p=0.404), or male size (GLM, χ_1^2=1.211, p=0.271). Consecutively, winged females were more often attempted (χ_1^2=73.026, p<0.000) and copulated (χ_1^2=85.247, p<0.000) than their wingless sisters: the majority of winged females were attempted and mated, whereas only a few wingless females were attempted (N=6), and although they were never observed to reject in response, none were copulated (Fig. 5). The probability of a female being attempted did

not depend on their size or the male's size, and neither of these factors nor rejection significantly influenced chances of copulation.

Choice between winged females with one wingless allele or without

The control experiment using winged beetles with the wild-type (WT) and the heterozygote (HET) genotype on the wingless locus revealed no differences in behaviour between the genotypes in either sex with respect to the frequency of interactions and the timing of behaviour, except that WT males spent generally less time in palpation of the female that was eventually mated (GLM, $F_{1,56}$=5.323, p<0.050).

Choice between winged females with two wingless alleles or without

Males were offered two winged females, one with the wingless genotype (WL_F), and one with the wild-type genotype at the wingless locus (WT). Females did not differ in size (t-test, t=0.210, p=0.835). When both females genotypes were palpated in a trial, both were equally likely to be palpated first by the male (χ_1^2=2.286; p=0.131). There was no significant difference in the total number of trials where palpation occurred (χ_1^2=0.970, p=0.325), but attempts to mate were fewer with wingless females than with wild types (χ_1^2=7.811, p<0.01), and they were less often copulated (χ_1^2=10.125, p<0.01) (Fig. 7). Both female genotypes responded equally often with rejection behaviour upon male attempt (WT: 10/27; WLF: 1/10; χ_1^2=1.423, p=0.233), but rejection never prevented mating. The timing of behaviour preceding mating did not differ significantly between the female genotypes.

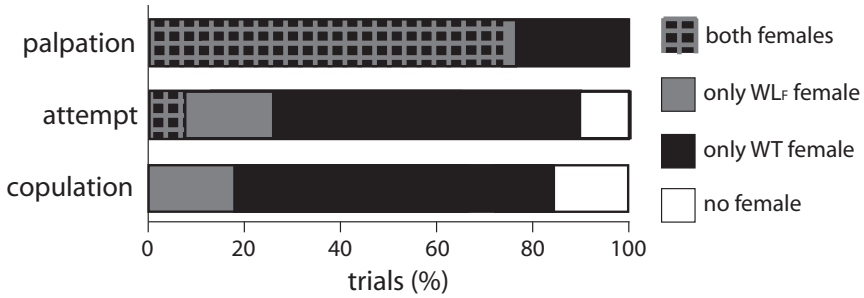


Figure 7. Percentage of trials in which wild-type males palpated, attempted, and copulated winged females with the wild-type genotype (WT) and the wingless genotype (WL_F), when offered simultaneously in a choice test (N=38).

Discussion

Winglessness affects mating in *A. bipunctata*

We observed strong effects of winglessness on mating in *A. bipunctata.* Specifically, winglessness in females affected male mating behaviour. Thus, males offered a single conspecific female never copulated with her when she had no elytron tissue (WL_0), but attempts and copulations increased with female elytron length, and fully winged but genetically wingless females (WL_F) were as often copulated as winged wild types (WT) (Fig. 3). When given the choice between a winged and a wingless sister, successful males always copulated with the winged one (Fig. 5). When offered two winged females, one genetically wingless and the other wild-type, the latter were more frequently copulated (Fig. 7).

We found no evidence for pleiotropic effects of the wingless allele on cues for mating that could explain these results. First, our behavioural essays revealed no effects of the wingless allele on mating behaviour in either sex: males and females possessing one or two copies of the wingless allele behaved similarly to wild types. This also justifies the use of winged beetles heterozygous for the wingless trait instead of wild types in choice experiments 1 and 2. Second, we found a pleiotropic effect of the wingless allele on body size, a trait that has been associated with mating preference (Tomlinson et al. 1995; Yasuda and Dixon 2002). Wingless beetles were on average larger than winged conspecifics. However, female size was never found to affect the chance a female was mated significantly. Third, a preliminary analysis of the composition of cuticular hydrocarbons from the pronotum, elytra, and abdomen of winged and wingless females revealed that only 4% of the total variation was explained by the different genotypes, whereas 40% was explained by variation between body parts. These results indicate that winged and wingless morphs differ hardly in CHC composition. This is in line with the hypothesis that these CHCs act as species-specific recognition cues (Hemptinne et al. 1998), and not as recognition cues of genotypes within species.

In sum, the wingless allele does not seem to affect behavioural, visual or olfactory mating cues. It is, therefore, likely that the observed effects of winglessness in females on male mating behaviour can be entirely attributed to the deviations in the female elytra. Below we initially discuss which mating cues provided by the elytra can explain the reduced mating frequency with wingless females. We then examine implications for wingless beetles in the wild and for their use as natural enemies in biological control programs.

Elytra and chemical mating cues

Although elytra bear CHCs that act as species-specific recognition cues eliciting male mating behaviour (Hemptinne et al. 1998), we reason that the lack of elytra tissue does not impair species recognition since it is unlikely that the chemical mating cues are associated exclusively with the elytra. First, the insect literature reports that CHCs are part of the cuticular waxes that cover

entire insect bodies (Howard and Blomquist 1982). Second, we analysed the frequency with which different body parts were palpated, and how often this behaviour was followed by copulation. Whereas Hemptinne et al. (1998) noticed that elytra were often contacted first by males, we found that initially in courtship, the anterior portion of females was generally palpated more frequently than the posterior. Palpation on the front of the female body was as frequently followed by copulation attempts as palpation on the elytra of winged and fully-winged wingless females. This implies that the front of the body also includes the species recognition cue(s) that elicits copulation attempts. Our observation that palpation frequency within a trial increased with shorter female elytron length is probably a direct consequence of the decreasing chance of being mated, and not a sign of lack of recognition. The less often, or later an attempt is made to copulate, the more time is spent on palpation. Third, in a preliminary analysis of the CHC composition in *A. bipunctata*, CHC composition of the elytra did not significantly differ from that of the abdomen (unpublished data). When males palpate the dorsal surface of wingless females, they may contact the abdominal surface instead of the elytra but these body parts probably provide the same chemical cues. Research on other species of beetle indicates that males will mate with any material, as long as they bear the active compound (Fukaya et al. 1996; Yasui et al. 2003; Sugeno et al. 2006).

In summary, we assume that body parts other than the elytra can provide the olfactory species recognition cue, and therefore elicit mating behaviour in *A. bipunctata*. We then assume that wingless females will be recognized as potential mating partners upon palpation. To confirm this, the active compound(s) in the CHC blend will need to be identified for *A. bipunctata* (Howard and Blomquist 2005), and its presence and abundance compared between winged and wingless specimens.

Elytra and visual mating cues

For *A. bipunctata*, data on visual mating cues potentially provided by elytra are scarce. Hemptinne et al. (1996) showed that contact with female elytra of *A. bipunctata* induced intensified searching behaviour by *A. bipunctata* males. In contrast, such elytra washed in chloroform, did not elicit this response. Female elytra of the ladybird *Coccinella undecimpunctata* (L.), similar in size and colour to those of *A. bipunctata*, also failed to elicit this response. This implies that contact sex pheromones on elytra may be more important than visual cues. Studies of certain populations and laboratory stocks have suggested that female preferences can occur for male melanic colour morphs in *A. bipunctata* (Majerus et al. 1982). However, such preference may not be mediated by the colour pattern itself since the different colour morphs differ in many other traits (De Jong et al. 1996). Moreover, this form of preference has not been found for the Utrecht localities (Brakefield 1984c). In our choice experiment with winged and wingless sisters, only winged females were copulated although they were not any more likely to be the first to be palpated by the male. This strongly suggests that the shape of the elytra is not a visual cue for species recognition or mating in this species.

Elytra and tactile mating cues

Our behavioural assays strongly indicate that the female elytra provide a tactile cue for male mating behaviour in *A. bipunctata*. We put forward three potential mechanisms that are not mutually exclusive. With a few exceptions, all wingless females palpated were mounted, so elytra do not seem a prerequisite for mounting. However, once mounted, males retracted their genitalia again frequently when attempting to copulate with a wingless female, but this was seldom observed with winged females. The chance that a male attempts to copulate with a wingless female increased with increasing elytron length (Fig. 3). In the no-choice experiment, wingless females in DWL class 5 with more than ¾ of the abdomen length covered by the elytra were attempted as frequently as fully winged wingless females and wild types, but were copulated less frequently than either of the latter in the no-choice experiment. There was also a non-significant trend for decreased time from palpation to attempt, and from attempt to copulation, with increasing elytron length.

There is no evidence that male *A. bipunctata* express mate preference. Thus, it is more likely that these observations are related to the ability for males to mate with particular phenotypes, and the properties of the elytra that could facilitate this in a physical way. Our first hypothesis is that the outline of the elytra guides a mounted male to the precise location of the female genitalia. The female genitalia are located at the tip of the abdomen, and in wild types, close to the caudal end of the elytra (Fig. 8). This is further supported by observations of some males in this study trying to insert their genitalia underneath the pronotum shield and the end of the truncated elytra of wingless females halfway along the abdomen. In some other species of beetles, mating males have been shown to use tactile cues for orientation. Males of a longicorn beetle positioned themselves on female models based on the shape and size of the model (Fukaya et al. 1996), and males of a staphilinid beetle (Peschke 1979), and a anobiid beetle (Ward 1981) were found to use female setae for orientation. Second, elytra could provide the male with grip while mating. This was also as observed in *Pidonia* longicorn beetle species (Tanigaki et al. 2007). Indeed, mating *A. bipunctata* males were sometimes observed to hold the edges of the female elytra (Fig. 8), for example when they could not stabilize themselves because there was no platform for their most caudal pair of legs to grip.

Figure 8. A wingless male copulates with a winged female while holding the edge of her elytra.

Mating by wingless *A. bipunctata* in the wild

We here consider the implications of our findings for the consequences of the wingless allele for mating in the wild. Wingless phenotypes rarely occur, but the wingless allele could be maintained for some time in winged individuals heterozygous for the wingless locus. Our results demonstrate that mating is not affected in such heterozygotes. Therefore, the wingless allele will only affect mating in homozygotes with a wingless phenotype. In our experiments with phenotypically wingless individuals, winglessness in females, but not in males, obstructed mating. However, all experiments were carried out in a restricted area preventing flight. Under natural conditions where flying is not hindered, the ability to fly will strongly affect the probability of mating of both sexes. Flight might be required to migrate to sites suitable for reproduction, since beetles switch between several different host plants throughout the season (Brakefield 1984a). Thus, wingless females might have difficulties finding or reaching areas with plants hosting aphid prey, and wingless males might have the same problem in localising and reaching these areas with potential mating partners. We, therefore, expect wingless beetles of both sexes to have a lower chance of mating with in the wild than their winged conspecifics. If males contact wingless females in the wild after all, it is difficult to predict the chances of copulation. On the one hand, winged females are likely to be favoured when both phenotypes are present at the same site. Therefore, we expect that female density is an important factor in determining the chance that wingless females are mated. On the other hand, we expect females to be more effective in rejecting males in open environments in nature than in our experiments where rejection rarely prevented males from copulation. For example, flipping over might then result in dropping off the host plant and release of the male, and winged females will consequently be able to flee from mating by flying away. In this respect, once localized by males, wingless females might be easier to mate with.

The evolution of winglessness in *A. bipunctata*

How does mating affect the evolution of winglessness in *A. bipunctata*? Mating frequency is a key factor in sexual selection, so if wingless individuals in the wild indeed mate less frequently than winged ones as suggested above, this may affect the evolution of the wingless allele. *Adalia bipunctata* is highly promiscuous (Haddrill et al. 2008). Polyandry is shown to increase offspring number in *A. bipunctata* (Haddrill et al. 2007), and is generally considered advantageous by increasing offspring quality (e.g. Zeh and Zeh 1997; Jennions and Petrie 2000). Wingless females, if indeed mating less frequently, may have a disadvantage in this respect.

However, female resistance behaviour suggests that mating is costly in *A. bipunctata* (Perry et al. 2009). Though no direct cost of mating in terms of reduced longevity was found for females mating multiply (Perry and Rowe 2008), other direct or indirect costs could play a role. For example, indirect costs may include the risk of sexually transmitted diseases which can have a large impact

on population dynamics (Knell and Webberley 2004). The highly virulent parasitic mite *Coccipolipus hippodamiae* (McDaniel & Morrill) is common in *A. bipunctata*, lives underneath their elytra, and is predominantly transmitted during mating (Hurst et al. 1995; Webberley et al. 2004). It does not affect mating behaviour of its host (Webberley et al. 2002), but has severe fitness effects by causing female sterility and deleteriously affecting males, too (Hurst et al. 1995). The ladybird's promiscuity thus promotes sexual transmission of this parasite (Webberley et al. 2004; 2006). Wingless females may have a lower chance of becoming infected when mating less frequently, and by having less elytron tissue to host the mites. They could have a selective advantage in this context. Such benefits could partly outweigh the major disadvantages of winglessness (Ueno et al. 2004), and thus promote some local spread of the wingless allele in the population. To test this hypothesis, wild populations of *A. bipunctata* should be screened for the frequency of the wingless allele and the incidence of infection with the parasite, and laboratory experiments used to examine the relationship between elytron length and the transmission rate of parasites.

Implications for the use of *A. bipunctata* in biological control

Adalia bipunctata is sold as a biological control agent of aphid pests in greenhouses and urban trees. It has been shown that wingless morphs of this predatory ladybird can be more effective in aphid control because their residence time on the host plant is longer (Lommen et al. 2008). Ease of mass-rearing of wingless morphs is a prerequisite for commercial companies to consider them as a new product. Our experiments should not necessarily raise concerns for mass-rearing since the limited time frame in which the behaviour assays were performed has probably strongly affected the results. When wingless ladybirds are held in groups over a longer period, matings are observed frequently, and they reproduce well. This indicates female elytra facilitate, but are not essential for, mating.

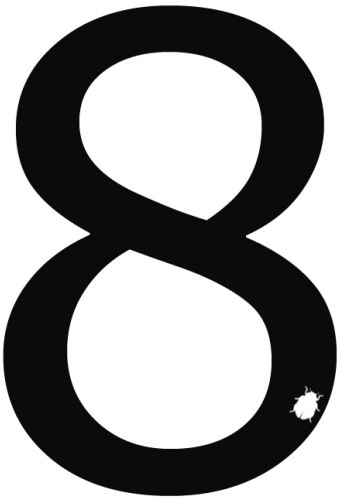
Acknowledgements

We are grateful to Gijs-Jan Vleming for performing pilot experiments, Bertanne Visser and Erik Kok for providing software to record and process mate behaviour. Kees Koops kindly provided help in rearing ladybirds, Koppert B.V. *Ephestia* eggs, and Olympus equipment for photography.

PART C

Synthesis and outlook

The logo of the Evolutionary Biology Group (a compilation of our study insects, made by Nicolien Pul), the group in 2007, and my office



Synthesis

Suzanne T. E. Lommen

In this thesis, I explore natural variation in wing length of the two-spot ladybird beetle, *Adalia bipunctata*, and exploit this knowledge to improve the effectiveness of this natural enemy for the biological control of aphids. I take a multidisciplinary, experimental approach to examine the causes and consequences of wing length variation in this species. The thesis includes genetical, developmental, behavioural, life-history, and population-dynamic studies. I discuss these results, on the one hand in an evolutionary perspective, and on the other, in the context of biological control.

In this chapter, I first provide a short background to the study system, the evolution of flightlessness, and ladybirds as biological control agents. I then summarize the experimental work presented in the previous chapters. Next, I integrate the results of this work to address the evolution of the wingless character in *A. bipunctata*. Following this, I discuss the potential for wingless morphs of *A. bipunctata* to improve the biological control of aphids compared to winged conspecifics, and propose how variation in wing length morphology might be further exploited to enhance the use of wingless beetles as biological control agents.

Background

Adalia bipunctata is a predatory ladybird beetle native to large parts of Europe and North America. This generalist species feeds on a wide range of aphid species. "Wingless" adult morphs of *A. bipunctata* occur naturally in at least one population in The Netherlands (Marples et al. 1993) and five in the United Kingdom (Majerus and Kearns 1989, M. E. N. Majerus personal communication), but they are rare. In the wingless morph both pairs of wings are truncated, and this malformation is regulated by a recessive allele at a single locus (Marples et al. 1993; Ueno et al. 2004). The frequency of the wingless allele in the field, is however, unknown. In laboratory populations fixed for this wingless trait, cryptic variation for the extent of wing reduction was revealed (Marples et al. 1993; Ueno et al. 2004). There is a continuous range of wingless phenotypes, from individuals lacking all wing tissue to those only missing a small piece of the tip, but the underlying mechanisms regulating this variation is unknown.

Flightlessness occurs in many species of insects, and flightless morphs typically have reduced or no wings. In some insect species, the evolution of flightlessness is considered to be adaptive, and thus to result from natural selection in its favour. This has often been explained by a trade-off between the ability to disperse by flight, and reproduction (Wagner and Liebherr 1992). However, among ladybirds (Coccinellidae), winglessness is uncommon (Pope 1977). In this thesis, I ask why winglessness, and variation in the degree of wing reduction, exists in *A. bipunctata*. On the one hand I test the causes of wing reduction and on the other, I examine the effects of wing reduction

and potential pleiotropic effects of the loci regulating wing length.

Predatory ladybirds are used for biological control of aphids by means of inundative augmentation, where large numbers of mass-reared natural enemies are released for immediate and short-term pest control (Eilenberg et al. 2001). To be cost-effective, the effects of biological control should outweigh the production costs. Currently, the application of *A. bipunctata* in biological or integrated control programs is restricted, one of the reasons being that the adults tend to fly away from the target host plants. This reduces the efficacy of control, and requires repeated releases, while mass-rearing is expensive. In some other species of ladybird, limiting their ability to fly has indeed enhanced their residence time on the host plants following release, and sometimes, their efficacy in biological control of the aphids (Ignoffo et al. 1977; Ferran et al. 1998; Tourniaire et al. 1999; Weissenberger et al. 1999; Seko et al. 2008). In this thesis, I test the potential for wingless morphs of *A. bipunctata* to improve the biological control of aphids compared to winged morphs.

Summary of experimental work

The research described concerns experimental work using laboratory populations of *A. bipunctata* of which all ancestors originated from a wild, Dutch population. The experimental work is divided into two sections. **Part A** (chapters 2-3) concerns the efficacy of wingless *A. bipunctata* as biological control agent of aphids. In **part B** (chapters 4-7), I explore causes and consequences of natural variation in wing length in *A. bipunctata*.

Biological control by wingless *A. bipunctata*

In **chapter 2** I provide the proof of principle that wingless morphs of *A. bipunctata* stay longer on aphid-infested plants after their release than do winged morphs, and that this can result in enhanced aphid control. However, whether or not control was improved, depended on the species of aphid used. We released a single ladybird, either winged or wingless, on single, caged pepper plants infested with either *Myzus persicae* or *Aulocorthum solani*. In control treatments, no ladybird was released. Ladybirds were monitored during 48 hours, and then the numbers of aphids remaining on the plants were counted. With both species of aphids, wingless ladybirds remained longer on the plants than winged ones. Nevertheless, both beetle morphs reduced the numbers of *A. solani* to a similar extent. This is likely to be explained by the tendency of A*. solani* to drop off the plant upon disturbance, which was probably invoked by the initial presence of a ladybird on the plant, independent of its phenotype. In contrast, numbers of *M. persicae* were only reduced by wingless morphs of *A. bipunctata*, probably because of the increased consumption as a direct effect of the prolonged residence time on the plant. In a control experiment where individual ladybirds were

fed one of these species of aphids, or a third one, on single leaves in Petri dishes, no differences in feeding behaviour were found between wingless and winged ladybirds. This indicates the wingless alleles have no pleiotropic effects on this behavioural aspect. Therefore, I conclude that wingless ladybirds can improve biological control of aphids, provided consumption of aphids is the main mode of aphid population reduction.

Chapter 3 provides proof of principle that the release of wingless *A. bipunctata* can reduce aphid-borne honeydew beneath urban lime trees. Honeydew is excreted by aphids, and spoils the surface beneath the infested tree canopy. This can cause problems and risks of accidents in urban environments, and therefore, many Dutch municipalities release commercially bred *A. bipunctata*. To my knowledge, this is the first time that this potential control measure has been evaluated scientifically. We released larvae and adults of our wingless laboratory stock in two different species of lime trees (*Tilia* spp.) in a region of Amsterdam after natural infestation by the lime aphid, *Eucallipterus tiliae* L.. Releases were made in eight consecutive weeks, and during that period and one month later, the amount of honeydew dripping from these treated trees was compared to untreated ones in a randomized block design. The amount of honeydew was measured by exposing water-sensitive papers beneath the canopy in a standardized way. The amount of honeydew was approximately halved in treated trees compared to controls. Because both larvae and adults of the wingless strain were released, it remains unclear whether the observed effects can be attributed to the flightless characteristic of this strain. It is also questionable whether this reduction is satisfactory for reducing the associated nuisance to the city population. Finally the applied method should be modified to become cost-effective.

Genetics of wing length variation in *A. bipunctata*

Chapter 4 shows that the genes regulating winglessness and melanization are physically linked on the same autosome. This was determined by phenotyping the offspring from families of parents varying in these characteristics of the elytra. This genetic linkage might facilitate the identification of loci regulating these traits.

In **chapter 5**, I develop an extended model for the genetic architecture of winglessness in *A. bipunctata*. The most parsimonious model contains three polymorphic autosomal loci, of which a single bi-allelic gene regulates the wing status with the winged allele dominant to the wingless one (Marples et al. 1993; Ueno et al. 2004), and two other polymorphic genes interact with the environment to regulate the expression of the wingless trait (chapter 5). The model for the expression of the trait resulted from a series of studies using methods of classical genetics. First, we revealed a strong family-by-temperature interaction on the variation in the extent of wing reduction by rearing offspring of 41 wingless families at two different temperatures. Heritability of the degree of wing reduction was 0.64±0.09 at 19°C and 0.29±0.06 at 29°C. Second, artificial selection on the degree

of wing reduction at 20°C over five generations demonstrated that the degree of wing reduction can be altered rapidly. Both extreme phenotypes (individuals without any wing tissue versus individuals resembling winged wild types) were obtained within a few generations by selection in opposite directions in two replicates. It was also noticed that the upward selection lines had a higher reproduction than the corresponding downward selected lines. Third, a pedigree analysis of 96 wingless families derived from these selection lines covering four generations indicated that the heritable component of the variation in wing reduction encompasses at least two polymorphic genes. The heritable variation in the degree of wing reduction observed in wingless morphs is cryptic in winged morphs, but has its origins in standing genetic variation in the wild population. I reason that this phenotypic variation in wingless *A. bipunctata* corresponds to an evolutionary model of gradual wing loss, which predicts that winged species evolve to become wingless by gradual loss of wing tissue over time. However, I recognize that such evolution is not likely to occur in *A. bipunctata* since evidence for the adaptive value of the wingless state is lacking. Nevertheless, our study illustrates the potential of developmental mechanisms for gradual evolution. Finally, I argue that the manipulation of wing length, as achieved in the artificial selection experiment, can be exploited to improve biological pest control by wingless ladybird beetles.

Development of wing length variation in *A. bipunctata*

In **Chapter 6**, I conclude that the development of the wings is delayed in the larval stage of wingless morphs resulting in incomplete wings at the time of pupation, and consequently, yielding adults with truncated wings. This was discovered by a series of morphological observations and measurements. Larval stages were examined histologically. Because wings begin to develop underneath the skin in these stages, the method was by necessity destructive. Therefore, wingless families that differed in their degree of wing reduction were reared, and their offspring compared to individuals from the wild type stock. After pupation, the outline of the wings is visible externally from the outside, and therefore, the shape and size of wings could be compared within individuals both in the pupal stage and later in the eclosed adult. Morphometric measurements on adults showed that the degree of truncation is closely correlated between the two pairs of wings (elytra and the flight wings), suggesting a coupled developmental mechanism. However, the posterior part of the wing was, on average, reduced to a larger extent than the anterior part. Finally, gene expression studies in larval wing tissue using immunohistochemistry of some important candidate wing development genes known from *Drosophila* (*nubbin*, *engrailed*, and *Distal-less*), showed that the expression of *Distal-less* was incomplete in larvae of wingless beetles. This pattern generally corresponded to the truncation patterns observed in adult wing phenotypes: this gene is typically expressed along the entire wing margin, but expression was lacking in the distal tip, extending to a variable extent towards the posterior, and sometimes the anterior margin. Since *Distal-less* is part of the

dorso-ventral patterning gene cascade, I suggest that modifications in this patterning pathway may explain both the incomplete expression of this gene and the truncation in wings of adult wingless *A. bipunctata*. However, it is not understood how such developmental flaw is connected to the asynchronic development of wing tissue, and how this would yield truncated wings.

The role of elytra in mating, and consequences for wingless *A. bipunctata*

In **Chapter 7**, I argue that the female elytral structure plays a role in mating in *A. bipunctata* by means of tactile cues, and that therefore, the probability wingless females mating declines with decreasing length of their elytra. Mating behaviour experiments were performed in small Petri dishes where individual males were offered either one or two females (no-choice and choice experiments, respectively). Their behaviour was then recorded for fifteen minutes. Four types of ladybirds were used: phenotypically wingless individuals (homozygous recessive for the major wingless gene), and phenotypically winged individuals with either no (wild-type), one (heterozygous) or two wingless alleles (genetically wingless, but artificially selected for long elytra). Comparing the behaviour of these types provided no evidence for pleiotropic effects of the wingless allele on mating behaviour. Male-female interactions were strongly affected by the female phenotype, but not the male phenotype. The incidence of mating decreased with female elytra length in no-choice tests, and in choice testes with a winged and a wingless female, the latter were never mated. These studies were limited to a very short time frame, and I expect that the frequency of mating with wingless females will increase if males have no choice but are given more time. Thus, I am not concerned that winglessness will hamper mating in mass-rearing. In contrast, it is hard to predict the mating success of wingless individuals in the field, since flight can be important for males in finding mates, but may also be a means for females to escape mating.

Evolution of winglessness in *Adalia bipunctata*

I have examined causes and consequences of variation in wing length in *A. bipunctata* (part B). I will now use the results to discuss whether natural selection can explain the evolution of winglessness in the Utrecht population of *A. bipunctata*.

Causes of wing length reduction

I have discovered several mechanisms related to the development of reduced wings:

- The major locus regulating winglessness is linked to melanism (chapter 4).
- In addition to the major locus regulating winglessness (Ueno et al. 2004), at least two other polymorphic loci regulate the expression of this trait in our stock, as does environmental

temperature (chapter 5).

- In wingless morphs, the development of the wing tissue in the larval stages L3 and L4 is delayed, and as a result, the wing tissue is incomplete at the time of pupation, which then results in adults with truncated wings after metamorphosis (chapter 6). Within the wingless phenotype, the rate of larval wing development seems to be positively correlated to the length of the adult elytra (chapter 6).
- The wingless genotype is associated with a lack of expression of the gene *Distal-less* in the larval wing tissue (chapter 6).
- The wingless allele has no effect on the development of flight muscles (S. V. Saenko and S. T. E. Lommen, unpublished results).

A next step towards fully understanding the mechanistic causes of variation in wing length, would be to link the underlying genetics to the developmental mechanisms. In other words, to identify the loci regulating winglessness and variation in the extent of wing reduction, and to find out how these loci are associated with the development of the larval wing tissue. One approach could be to use the deviations in wing development to select candidate genes, and to consequently test their function in *A. bipunctata* by establishing RNA interference in the larvae, and phenotyping the emerging adults (Tomoyasu and Denell 2004). Another approach could be to use families segregating for the wingless phenotype for comparative genetic analysis by sequencing.

Consequences of the wingless allele and wing length reduction

In addition to the causes of winglessness, I have examined some putative consequences of the wingless genotype and phenotype in *A. bipunctata*. Table 1 list my findings together with existing knowledge. Among all the traits investigated, wingless morphs only performed better than winged morphs with respect to pre-oviposition period and adult size. However, it is unclear if wingless females will really start to lay their eggs earlier in their lives than winged conspecifics since their development takes longer (Table 1) (Ueno et al. 2004). An increased body size can be advantageous for both females and males. In many insects, female body size is very closely positively correlated to fecundity (Honěk 1993). However, total fecundity in wingless females was much lower than that of winged females, resulting from a reduced longevity (Table 1) (Ueno et al. 2004). Perry et al. (2009) showed that larger males of *A. bipunctata* can more easily overcome female resistance in mating, but they did not elucidate why larger males are more successful in that. Under conditions when body size is the key factor, wingless males might thus benefit from being rejected less frequently, but when this benefit is accounted for by body weight, there should be no benefit for them since pupal weights do not differ significantly between winged and wingless beetles (Table 1).

For two traits examined here, it is not clear how they would affect fitness in the field (Table 1). First, wingless beetles, like winged ones, develop flight muscles. However, since they cannot fly,

both the development of these muscles and their use in moving truncated wings (K.G. Koops & S. T. E. Lommen, unpublished results) seems a waste of energy. Muscles are costly to develop since they are rich in proteins. In some species this has been shown to come at the cost of reproduction. For example, in female *Gryllus firmus* crickets, the mass of the dorsolongitudinal flight muscles (the main flight muscles) is negatively correlated with ovary mass in females (Roff et al. 2002). It is hypothesized that in the evolution of wingless morphs, dispersal was first inhibited prior to a decrease in flight muscle development, and before the wings were finally reduced (Roff 1986). Examination of some wingless insects indeed confirms the (partial) loss of flight muscles (e.g. Jackson 1928; Smith 1964; Zera and Mole 1994). Second, female mating frequency of wingless females was lower than that of winged females in 15-minute long trials, but it is unlikely that these results can be translated directly to a field situation where individuals are not limited in space and time. Moreover, mating involves both benefits (reproduction), and costs (superfluous matings are costly (Perry et al. 2009), and mating involves the risk of sexually-transmitted diseases (Hurst et al. 1995)). Thus it is hard to predict how these results affect natural selection.

Finally, the fitness of wingless morphs is reduced compared to that of wildtypes with respect to the six remaining life-history traits, and flight ability (Table 1). In addition, removal of the elytra resulted in a higher predation rate of the ladybird beetle *Coccinella septempunctata* L. by a bird, which was explained by the aposematic function of the elytral colour pattern (Dolenska et al. 2009).

The evolution of winglessness in *Adalia bipunctata*

Overall, wingless phenotypes of *A. bipunctata* show a reduction in many important fitness traits, and this disadvantage probably largely outweighs any fitness benefits resulting from the few traits where they perform better. Sometimes, the existence of traits that are disadvantageous in homozygotes, can be explained by increased fitness of the heterozygotes. However, Ueno et al. (2004) suggest a negative pleiotropic effect of the wingless allele on the fitness of *A. bipunta* heterozygous for the wingless trait. In conclusion, these results indicate the wingless allele will be selected against by natural selection (Ueno et al. 2004).

This is not surprising, given that this wingless genotype in *A. bipunctata* corresponds to a reduction in both pairs of wings, and not only flight wings. In other flightless beetles typically only the flight wings have been reduced (Darlington 1936; Smith 1964; Dybas 1978; Roff 1990), whereas the elytra are normally not changed in size (but see Dybas 1978), only sometimes have become fused or rigidly locked (Darlington 1936; Smith 1964). Elytra fulfill an important role in the protection of ladybirds against predators and adverse environments (e.g. they protect against dessiccation at low humidity) (Crowson 1981), and play a role in mating behaviour. Indeed, in wingless *A. bipunctata*, lacking both flight wings and elytra, not only flight is impaired, but also many fitness traits and mating behaviour are affected (Table 1). Thus, generally, there seems to be a strong constraint on

the loss of elytral tissue. In addition, wingless *A. bipunctata* exhibit fully developed flight muscles, which are costly to develop but do not even contribute to an increased dispersal.

The wingless allele in the Utrecht population has presumably been maintained by other evolutionary processes: a balance between recurrent mutation (explaining the origin of the wingless allele) and selection (negative natural selection explaining the disappearance of the wingless allele), or by genetic drift. Mutation hotspots are commonly observed in genomic sequences and may explain recurrent mutations (Maki 2002; Tian et al. 2008). A recent mutation-accumulation study in *Caenorhabditis elegans* Maupas shows that some phenotypic variants are much more often induced by mutation accumulation than could be expected, and this was explained by developmental processes (Braendle et al. 2012). Genetic drift is a stochastic processes, and is more likely to occur in small populations (Gillespie 2006).

To evaluate the likelihood of these alternative evolutionary processes as an explanation for the existence of the wingless allele in *A. bipunctata*, population genetic studies should be conducted among several populations, preferrably including others where winglessness was found. These can estimate the frequency of the wingless allele and the size of these populations, which can then be used to test predictions related to the different processes. At present, the evolution of winglessness in *A. bipunctata* remains puzzling.

Table 1. The performance of wingless beetles compared to winged morphs with respect to several important fitness traits. For each trait, the second column indicates how wingless phenotypes perform compared to winged ones, and the third column indicates the prediction whether that would result in lower (-), equal (=), of higher (+) fitness in the field.

trait	**performance of WL compared to W**		**reference**
larval development time	longer	-	Ueno et. al 2004
pupal development time	longer	-	Ueno et. al 2004
juvenile mortality	higher	-	Ueno et. al 2004
pupal weight	equal	=	Ueno et. al 2004
adult size	larger	+	M. E. van Schoor, S. T. E. Lommen, P. W. De Jong & P. M. Brakefield, unpublished results
development of flight muscles	equal	?	S.V. Saenko & S. T. E. Lommen, unpublished results
flight ability	lower (typically not at all)	-	S. T. E. Lommen, K. G. Koops, P. W. De Jong & P. M. Brakefield, unpublished results
pre-oviposition period	shorter	+	Ueno et. al 2004
oviposition period	shorter	-	Ueno et. al 2004
adult longevity	shorter	-	Ueno et. al 2004
female aphid consumption behaviour	equal	=	chapter 3
female mating frequency	lower	?	chapter 7
male mating frequency/behaviour	equal	=	chapter 7
total female fecundity	lower	-	Ueno et. al 2004

The potential of wingless *A. bipunctata* in biological control of aphids

Efficacy in aphid control

In this thesis, I examine the effect of the release of a wingless strain of *A. bipunctata* on aphid control (Part A). For the first time, I show that naturally wingless morphs of *A. bipunctata* have longer residence times than winged conspecifics after release on to aphid-infested plants, which can consequently result in better control (chapter 2). I also demonstrate for the first time that release of this wingless strain greatly reduces aphid-borne honeydew beneath urban lime trees (chapter 3). These results add to the growing body of evidence that inundation of predatory ladybirds with impaired flight ability can help to control aphid pests. I argue that the use of natural wingless morphs of *A. bipunctata* may be preferred over the use of exotic species in areas native to *A. bipunctata*.

We have found no pleiotropic effects of the wingless allele on female consumption behaviour (chapter 2) or male mating behaviour (chapter 7). Therefore, wingless beetles seem to behave in a similar way to wild types, and the comprehensive knowledge about behaviour of *A. bipunctata* (Wratten 1976; Hemptinne et al. 1996; Omkar and Pervez 2005; Hodek et al. 2012) can probably also be applied to wingless morphs.

To further assess the potential for wingless *A. bipunctata* in biological control, their efficacy should be investigated in more detail in relation to the timing and frequency of release, the numbers and stages released, and the way they are distributed in the crop. Their dispersal range is likely to be smaller than that of conspecifics capable of flight, which has implications for the location and the number of points of release in the crop. However, wingless adults can disperse effectively in the crop by walking (Tourniaire et al. 1999, S.T.E. Lommen and K. G. Koops, unpublished results). It is unlikely that releasing them is more costly than in current practice, where larvae are released in hotspots of aphids (J. van Schelt, Koppert B.V., personal communication).

Such experiments to refine the application should be carried out on a large scale. To examine whether the release of flightless beetles will result in reproduction in the crop, and whether another generation can consequently prevent the establishment of new aphid infestations, more detailed behavioural and long-term population dynamics studies should be conducted after release. In order to assess the potential benefit of wingless beetles over wild types, future studies should include comparisons with the release of winged beetles from the same source population and a similar history in the laboratory. Finally, the interactions between the plant species, aphid species and the ladybird (e.g. aphid defence behaviour, suitability of aphid species as prey related to host plant, accessibility of host plant for the ladybird) should be considered before applying wingless or winged *A. bipunctata* for biological control (Gurney and Hussey 1970; Jalali and Michaud 2012).

Costs of mass-rearing

Another requirement for the cost-effective use of wingless *A. bipunctata*, is the feasibility of cheap mass-rearing. I experienced that handling flightless ladybirds is much easier than handling those capable of flight, which saves costs of labour. However, based on previous studies on wingless *A. bipunctata*, producers of natural enemies have raised concerns regarding the reduced fitness of wingless *A. bipunctata* with respect to mass-rearing (J. van Schelt, Koppert B.V., personal communication). Indeed, Ueno et al. (2004) showed that wingless morphs have a longer development time, and a reduced life span, compared to winged conspecifics. As a consequence of their shorter lives, but a similar daily egg production (H. Ueno, unpublished results), their lifetime reproduction is also much lower. In addition, I discovered that wingless females were less likely to be mated than winged ones in fifteen-minute trials (chapter 7). However, both studies recognize that there is wide variation in the performance among wingless ladybirds with respect to these traits, and that those morphs with longer elytra generally perform better than those with shorter ones. Ueno et al. (2004) note a trend for wingless beetles with longer elytra to perform better with respect to developmental traits, and I demonstrate that the probability of being mated increases with elytron length within wingless beetles (chapter 7). I have also shown that this variation in elytron length is highly heritable, and can yield rapid responses to artificial selection (chapter 5). Therefore, I expect lines of wingless *A. bipunctata* artificially selected for increased wing length to be most suitable for mass-rearing. I show that two replicates of such selected lines indeed had a significantly higher reproduction than their corresponding lines selected in the opposite direction (chapter 5). The performance of selected lines in mass-rearing should be further verified experimentally. I have also demonstrated that the rearing temperature during juvenile stages affects the degree of wing reduction (Lommen et al. 2005; chapter 5). Therefore, choosing specific temperatures can further enhance the production of desired wingless phenotypes. The use of flightless lines with slight reductions of the wings may additionally improve aphid control further, because of an increased longevity and improved protection to desiccation. This should also be tested in biological control experiments. Selection on a morphological trait such as wing length that is correlated with life-history traits and efficacy in biological control is much easier and faster than selecting on these traits directly.

Generally, the rearing of ladybirds is considered costly because of their carnivorous diet and their cannibalistic behaviour. However, advances have been made in the development of alternatives to aphids as food for commercially reared *A. bipunctata*, which do not compromise the development and reproductive performance of the beetles (De Clercq et al. 2005; Jalali et al. 2009; Bonte et al. 2010; Jalali et al. 2010). Such diets could provide a cheaper and a more stable supply of food for the species, but confer the risk of modified foraging behaviour when beetles are subsequently released on aphid-infested crops (Ettifouri and Ferran 1993). In addition, releasing the larval stage instead of adults renders less costly rearing, and is common practice for *A. bipunctata*.

Risks and solutions

If wingless strains artificially selected for long wings are going to be used for biological control, it is recommended to attempt to reduce the loss of genetic diversity. Ongoing selection for increased wing length in wingless morphs might finally restore flight ability (chapter 5). Therefore, I advise selecting for morphs that are still visually slightly distinct from wild type beetles. This would also ease segregation of phenotypes after outcrossing to wild types. Alternatively, it may be possible to uncouple the length of the elytra and the length of the flight wings by artificial antagonistic selection, and thus obtain lines with strongly truncated flight wings but slightly truncated elytra. Such antagonistic selection managed to uncouple the size of the forewings from that of the hind wings in the butterfly *Bicyclus anynana* (Butler 1879), resulting in butterflies with larger fore wings than hind wings, and vice versa (Frankino et al. 2007). Whether such modification in allometry is possible in *A. bipunctata* depends on the underlying developmental mechanisms.

The release of genetically wingless *A. bipunctata* in agricultural systems or in ornamental trees exposes them to wild populations of *A. bipunctata*. Therefore, there is a chance of mixed mating, resulting in an increased frequency of the wingless allele in natural populations. However, according to our current knowledge, wingless morphs have a disadvantage in many fitness traits, and no heterosis was found (Ueno et al. 2004). Therefore, I expect the wingless allele to be selected against by natural selection, and finally to disappear from natural populations.

Finally, since ladybird beetles are popular, the public may react adversely to the use of 'handicapped' specimens. The use of wingless lines artificially selected for long elytra also reduces this cosmetic problem.

Realizing a commercial wingless stock

Summarising the above, I argue that intraspecific natural variation in wing length of *A. bipunctata* can be exploited to improve the performance as biological control agent. Should extended research confirm the potential for commercialization, I suggest developing a "wingless" strain consisting of beetles with only slight reductions in wings. This process would encompass two levels of selection. First, the qualitative wingless trait should be fixed in a laboratory population of *A. bipunctata*. In principle, this only requires a single copy of the wingless allele, and two generations of rearing. Second, selection on the quantitative expression of the trait should be performed to obtain a "wingless" population with only slightly truncated wings. More generations of selection are required to fix this trait in the population (chapter 5). To prevent detrimental inbreeding effects, the numbers of individuals sampled as the initial population should be large, and the laboratory populations should be large, or outcrossed regularly to freshly sampled wild types (Wajnberg 1991; Bartlett 1993; Nunney 2003).

Since the wingless allele is recessive, and variation of wing length is cryptic in wild types (chapter 5),

the identification of the loci underlying these traits would allow developing markers to facilitate the screening of the desired genotypes in natural populations. The rapid development of molecular tools in general, and the upcoming sequencing of the genome of the ladybird *Harmonia axyridis* Pallas, would be helpful for identification, as well as the linkage between the loci regulating winglessness and melanism (chapter 4), and the recognition of some developmental processes that deviate in wingless morphs (chapter 6) may be helpful for a candidate gene approach.

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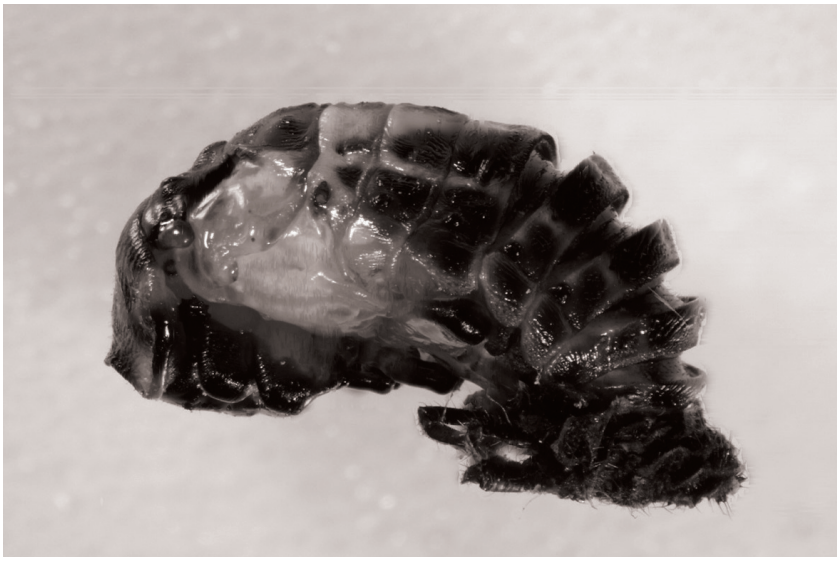

Pupa of a wingless two-spot ladybird beetle

A perspective on selective breeding to improve biological control agents

Suzanne T. E. Lommen

The selection of desired wing phenotypes of *A. bipunctata* as described in this thesis, boils down to selective breeding. This is a historical method widely used in plant and animal breeding, including some insects (e.g. bees and silk moths). Nearly a century ago, Mally (1916) already suggested to use genetic selection to improve natural enemies for biological control. Natural enemies have now been commercially produced as biological control agents for about a century, and the production has been professionalized over the past decades (Van Lenteren 2003b). However, intrapopulation diversity is still understudied (Wajnberg 2004), and selective breeding is little applied in this field (Hoy 2002).
Nevertheless, there is ample variation between and within populations of natural enemies for traits likely to be important in biological control (e.g. Bakker et al. 1993; Hopper et al. 1993; Lozier et al. 2008; Nachappa et al. 2010; Tabone et al. 2010; Wajnberg 2010; Wajnberg et al. 2012). Selective breeding is, therefore, potentially a powerful tool to select new or improve existing natural enemies (Hoy 1986; Narang et al. 1993; Nunney 2003). It is especially suited for augmentative control in controlled and closed environments such as greenhouses, and especially for species which have extensively been studied for their ecology (Hoy 1986), such as some ladybird beetles, predatory mites, and parasitoids.
Indeed, several studies report successful artificial selection of desired traits. Examples include the resistance for chemical pesticides in predatory mites and parasitoid wasps (Hoy 1986; Rosenheim and Hoy 1988; Johnson and Tabashnik 1993), drought and temperature tolerance in predatory mites and entomopathogenic nematodes (Hoy 1985; Shapiro et al. 1997; Strauch et al. 2004; Salame et al. 2010; Anbesse et al. 2012), and sex ratio in parasitoids (Hoy and Cave 1986; Ode and Hardy 2008). Some of these selected lines have proven successful in biological control after release (Hoy 1986; Hiltpold et al. 2010). There are few examples of such strains that have become commercially available, including a few predatory mites with enhanced pesticide resistance (Hoy 2002), and others that lost diapausing after artificial selection on this trait (Van Houten et al. 1995). However, in most cases the efficacy of the selected strains in biological control was not tested in the field or greenhouse (Hoy 1985). Future research should accumulate evidence that the effectiveness in the field can indeed be increased by selective breeding.

Previous objections to selective breeding

In the 1980s, “genetic improvement” of natural enemies was debated. Genetic improvement covers selective breeding but also hybridization and genetic engineering (the latter two lie outside the scope of this thesis). In a review, Hoy (1986) has listed a number of reasons why she thinks producers of natural enemies have been slow in picking up genetic improvement tools for improvement of their products: (1) the fear that laboratory adaptation would erode genetic

diversity and lead to decreased fitness in the field; (2) a lack of genetic knowledge, and (3) a negative image due to some previous failures. She also mentions the high costs involved in this procedure (4), because of the labour-intensive development. I think that by now, the objections can be mostly overcome.

For example, the loss of genetic variation (objection 1) is a general risk for captive populations (Mackauer 1976), and there are several ways to reduce the loss of genetic diversity. These include starting with a large population, keeping high numbers during breeding, outcrossing events, hybridization of strains, and crossing inbred lines (Wajnberg 1991; Bartlett 1993; Hoekstra 2003; Nunney 2003). If genetic erosion would result in lower fitness of the natural enemies, this will be detected sooner when recently developed quality control guidelines are applied (Leppla 2003; Van Lenteren et al. 2003). These guidelines include the regular measurement of many fitness components. It has even been suggested to develop "quality assurance" rather than "quality control" in biological control, which should guarantee the quality of commercially produced natural enemies rather than detect qualitative flaws that need to be corrected for (Bolckmans 2003). This trend should reduce the fear of genetic erosion by artificial selection (objection 1).

The recent advantages in the field of genetics and genomics will also render the process of selective breeding less labour intensive. Molecular tools can help to unravel the genetic architecture of traits (objection 2), including the identification of loci regulating these traits. Recently, genetic regions underlying several traits with relevance to biological control have been identified in natural enemies, including parasitoid host preference (Desjardins et al., 2010), clutch size, and sex ratio (Pannebakker et al. 2011). The latest developments in genomic and sequencing technology, will render the localization of the genetic basis of such traits more affordable and common practice. Once loci have been identified, molecular markers can be developed to facilitate screening of traits lacking morphological phenotypes. This can be both used in an inventory of the natural variation for these traits in the field, and of the individuals used in the breeding program ("marker-assisted selection")(Ribaut and Hoisington 1998; Dekkers and Hospital 2002). Especially for life-history or behavioural traits, this could save a lot of time (objection 4).

I am not aware of the current image of "genetic improvement" (objection 3), but I think that exploiting natural variation by selective breeding will be more easily accepted than genetic engineering by the public, and the natural character of the method can even be used in promotion. Biocontrol researchers might also need to put more effort in to promoting their positive results (Van Lenteren 2012).

Finally, when the benefit gained from the enhanced phenotype is large enough, this can outweigh the costs (objection 4). Cost-benefit analyses are scarce (Hoy 2002), but one of the studies estimated that the production costs of an egg parasitoid are reduced to a third when the sex ratio is modified in favour of the number of females (Irvin and Hoddle 2006).

Why is selective breeding timely?

Although the potential for selective breeding, or more generally, genetic improvement, of natural enemies has been highlighted in the past (Hoy 1986; Hopper et al. 1993; Narang et al. 1993), it is still hardly exploited in the development of biological control agents (Hoy 2002). I think there are several reasons why this method currently deserves a boost.

First, the practice of biological or integrated control of arthropod pests in agriculture is growing, and so is the number of commercially available natural enemies (Van Lenteren 2003c, 2012). This trend is likely to continue with increasing legal limitations on the use of pesticides. However, there are still pests that are currently not sufficiently controlled, including emerging pest species. New biological control agents should be developed, or existing species improved, that match with current integrated pest management strategies to control these pests. In recent decades, the commercial production of natural enemies has been professionalized (Van Lenteren 2012), and the field of genetics and genomics has made considerable progress. Altogether, there is more money, knowledge, and market for the implementation of new methods in the development or improvement of natural enemies for augmentative biological control.

Second, natural enemies are traditionally selected from the natural environment of the insect pest. As many insect pests originate from other countries, this frequently involves the import of non-native insect material which can involve risks for local biodiversity (Roy and Wajnberg 2008; De Clercq et al. 2011). In addition, recent international regulations impede the use of exotic species for biological control. The Convention on Biological Diversity (see www.cbd.int) hampers the export of natural enemies for biological control (Cock et al. 2010; Van Lenteren et al. 2011), whereas the FAO guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms demands a critical evaluation of species imported (Secretariat of the International Plant Protection Convention, 2005). These developments are likely to increase the need to explore and exploit natural variation in native natural enemies. There is already a trend towards preferentially utilizing indigenous natural enemies, with the indigenous natural enemies introduced to the European market outnumbering the exotic ones this century, whereas a reverse pattern was observed for the past century (Van Lenteren 2012).

How to exploit natural variation?

While very promising, selective breeding of biological control agents should only be used when no better naturally adepted species is available and safe for biological control. Therefore, both interspecific and intraspecific genetic variation should be evaluated when developing natural

enemies for biological control. When a species meets the requirements for biological control, this should be favoured over selective breeding of another sub-optimal species. For example, a strain of the parasitoid wasp *Aphitis lignanensis* Compere tolerant to extreme temperatures was developed (White et al. 1970) for areas of California with such climate, but was never applied because the species *Aphitis melinus* DeBach that was naturally adapted to such climate was established in the area (Nunney 2003).

Improving natural enemies for biological control by means of selective breeding requires a good characterization of intraspecific genetic diversity for the traits of interest (Narang et al. 1993; Wajnberg 2010). Traditionally, populations from different geographical locations are compared (Wajnberg 2004). Although these may differ strongly in traits, these populations do not always represent 'evolutionary significant units'. For example, Lozier et al. (2008) found that genetic distance did not correspond very well to geographical distance, and that large genetic variation existed within geographical areas in a parasitoid wasp. In another parasitoid wasp, Vink et al. (2012) showed that genetic variation was closely associated with the host taxon, but not to geographical location. It is, therefore, recommended to collect large numbers of specimens from several locations and host plants, to capture the majority of genetic variation in the wild as the basis for selection. Then, intra-population heritability of traits should be assessed to predict the response to selection (Falconer and Mackay 1996; Wajnberg 2004). When selective breeding succeeds in improving the trait of interest, it should be tested whether this is indeed translated into improved mass-rearing or biological control efficacy.

Case study of *Adalia bipunctata*

In this thesis, I have shown that aphid control by *A. bipunctata* can be improved by artificial selection of a naturally occurring wingless genotype (chapter 2), and that selection on the expression of this trait (chapter 5) may enhance its suitability for mass-rearing (chapter 8). Variation in wing length is, however, not the best example to generalise the potential usefulness of intra-population diversity for biological control agents, because the wingless phenotype seems to be unique and appears not to be adaptive (chapter 8). However, variation in many other traits is adaptive and more common, and moreover is potentially of interest for biological control.

For example, colour polymorphism is common in *A. bipunctata*, where dark (melanised) and red (typical) morphs coexist within populations (Brakefield 1984a). Because of the thermal properties of the melanisation, melanic morphs heat up in solar radiation earlier and are more active than typica morphs, at cool temperatures (De Jong et al. 1996). Therefore, melanic morphs might be more effective in biological control in areas with lower temperatures, such as greenhouses with

lower temperatures, and outdoors in cool climates (chapter 3). Since colour polymorphism is entirely under genetic control in this species (Majerus 1994), it can easily be fixed in mass-reared populations.

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References

Abouheif E (2004) A framework for studying the evolution of gene networks underlying polyphenism: insights from winged and wingless ant castes. In: Hall BK (ed) Environment, development, and evolution. MIT Press, pp. 125-137

Abouheif E, Wray GA (2002) Evolution of the gene network underlying wing polyphenism in ants. Science 297:249-252

Adachi-Hagimori T, Shibao M, Tanaka H, Seko T, Miura K (2011) Control of *Myzus persicae* and *Lipaphis erysimi* (Hemiptera: Aphididae) by adults and larvae of a flightless strain of *Harmonia axyridis* (Coleoptera: Coccinellidae) on non-heading Brassica cultivars in the greenhouse. BioControl 56:207-213

Agarwala BK, Dixon AFG (1992) Laboratory study of cannibalism and interspecific predation in ladybirds. Ecol. Entomol. 17:303-309

Anbesse SA, Strauch O, Ehlers R-U (2012) Genetic improvement of the biological control nematode *Heterorhabditis bacteriophora* (Rhabditidomorpha: Heterorhabditidae): heterosis effect enhances desiccation but not heat tolerance. Biocontrol Sci. Technol. 22:1035-1045

Arnaud L, Spinneux Y, Haubruge E (2003) Preliminary observations of sperm storage in *Adalia bipunctata* (Coleoptera : Coccinellidae): sperm size and number. Appl. Entomol. Zoolog. 38:301-304

Atallah J, Dworkin I, Cheung U, Greene A, Ing B, Leung L, Larsen E (2004) The environmental and genetic regulation of *obake* expressivity: morphogenetic fields as evolvable systems. Evol. Dev. 6:114-122

Bakker FM, Klein ME, Mesa NC, Braun AR (1993) Saturation deficit tolerance spectra of phytophagous mites and their phytoseiid predators on cassava. Exp. Appl. Acarol. 17:97-113

Bänsch R (1966) On prey-seeking behaviour of aphidophagous insects In: Hodek I (ed) Ecology of aphidophagous insects. Academia/Junk, Prague/The Hague, pp. 123-128

Bartelt RJ (2010) Volatile hydrocarbon pheromones from beetles. In: Blomquist GJ and Bagnères A (eds) Insect hydrocarbons. Biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, UK, pp. 492

Bartlett AC (1993) Maintaining genetic diversity in laboratory colonies of parasites and predators. In: Narang SK, Bartlett AC and Faust RM (eds) Applications of genetics to arthropods of biological control significance. CRC Press, Inc., Boca Raton, Florida, pp. 133-145

Beermann A, Jay DG, Beeman RW, Hülskamp M, Tautz D, Jürgens G (2001) The *Short antennae* gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila Distal-less* protein. Development 128:287-297

Bengtson SA, Hagen R (1977) Melanism in 2-spot ladybird *Adalia bipunctata* in relation to climate in Western Norway. Oikos 28:16-19

Blackman RL (1965) Studies on specificity in Coccinellidae. Ann. Appl. Biol. 56:336-338

Blackman RL, Eastop VF (1984) Aphids on the world's crops: an identification guide. Wiley-Interscience, Chichester

Blackman RL, Eastop VF (1994) Aphids on the world's trees. An identification and information guide. CAB International, Wallingford

Bolckmans KJF (2003) State of affairs and future directions of product quality assurance in Europe. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 215-224

Bonte M, Samih MA, De Clercq P (2010) Development and reproduction of *Adalia bipunctata* on factitious and artificial foods. BioControl 55:485-491

Boto L (2010) Horizontal gene transfer in evolution: facts and challenges. Proc. R. Soc. B 277:819-827

Bowsher JH, Wray GA, Abouheif E (2007) Growth and patterning are evolutionarily dissociated in the vestigial wing discs of workers of the red imported fire ant, *Solenopsis invicta*. J. Exp. Zool. (Mol. Dev. Evol.) 308B:769-776

Braendle C, Baer CF, Felix MA (2012) Bias and evolution of the mutationally accessible phenotypic space in a developmental system. Plos Genetics 6

Braendle C, Davis GK, Brisson JA, Stern DL (2006) Wing dimorphism in aphids. Heredity 97:192-199

Brakefield PM (1984a) Ecological studies on the polymorphic ladybird *Adalia bipunctata* in The Netherlands. 1. Population biology and geographical variation of melanism. J. Anim. Ecol. 53:761-774

Brakefield PM (1984b) Ecological studies on the polymorphic ladybird *Adalia bipunctata* in The Netherlands. 2. Population dynamics, differential timing of reproduction and thermal melanism. J. Anim. Ecol. 53:775-790

Brakefield PM (1984c) Selection along clines in the ladybird *Adalia bipunctata* in The Netherlands - a general mating advantage to melanics and its consequences. Heredity 53:37-49

Brakefield PM (1985) Polymorphic Mullerian mimicry and interactions with thermal melanism in ladybirds and a soldier beetle - a hypothesis. Biol. J. Linnean Soc. 26:243-267

Brakefield PM (2006) Evo-devo and constraints on selection. Trends Ecol. Evol. 21:362-368

Brakefield PM, de Jong PW (2011) A steep cline in ladybird melanism has decayed over 25 years: a genetic response to climate change? Heredity 107:574-578

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden P, Monteiro A, French V, Carroll SB (1996) Development, plasticity and evolution of butterfly eyespot patterns. Nature 384:236-242

Brakefield PM, Willmer PG (1985) The basis of thermal melanism in the ladybird *Adalia bipunctata* - differences in reflectance and thermal properties between the morphs. Heredity 54:9-14

Brisson JA, Davis GK, Stern DL (2007) Common genome-wide patterns of transcript accumulation underlying the wing polyphenisms and polymorphism in the pea aphid (*Acyrthosiphon pisum*). Evol. Dev. 9:338-346

Brisson JA, Ishikawa A, Miura T (2010) Wing development genes of the pea aphid and differential gene expression between winged and unwinged morphs. Ins. Mol. Biol. 19:63-73

Brown PMJ, Adriaens T, Bathon H, Cuppen J, Goldarazena A, Hagg T, Kenis M, Klausnitzer BEM, Kovar I, Loomans AJM, Majerus MEN, Nedved O, Pedersen J, Rabitsch W, Roy HE, Ternois V, Zakharov IA, Roy DB (2008) *Harmonia axyridis* in Europe: spread and distribution of a non-native coccinellid. BioControl 53:5-21

Brunetti CR, Selegue JE, Monteiro A, French V, Brakefield PM, Carroll SB (2001) The generation and diversification of butterfly eyespot color patterns. Curr. Biol. 11:1578-1585

Campbell G, Tomlinson A (1998) The roles of the homeobox genes *aristaless* and *Distal-less* in patterning the legs and wings of *Drosophila*. Development 125:4483-4493

Capinera JL (2008) Encyclopedia of entomology. 2nd edn. Springer, pp. 4346

Carroll SB, Gates J, Keys DN, Paddock SW, Panganiban GEF, Selegue JE, Williams JA (1994) Pattern formation and eyespot determination in butterfly wings. Science 265:109-114

Carter CI, Nichols JFA (1986) The lime aphid, *Eucallipterus tiliae*. In: Juniper BE and Southwood TRE (eds) Insects and the plant surface. Edward Arnold, London, pp. 342

Chan CX, Beiko RG, Darling AE, Ragan MA (2009) Lateral transfer of genes and gene fragments in prokaryotes. Genome Biol. Evol. 1:429-438

Chandler CH (2010) Cryptic intraspecific variation in sex determination in *Caenorhabditis elegans* revealed by mutations. Heredity 105:473-482

Cock MJW, van Lenteren JC, Brodeur J, Barratt BIP, Bigler F, Bolckmans K, Consoli FL, Haas F, Mason PG, Parra JRP (2010) Do new access and benefit sharing procedures under the Convention on Biological Diversity threaten the future of biological control? BioControl 55:199-218

Cohen SM (1993) Imaginal disc development. In: Bate M and Martinez Arias A (eds) The development of *Drosophila melanogaster*. Cold Spring Harbour Press, New York, pp. 747-843

Crawley M (2007) The R Book. John Wiley & Sons Ltd, Chichester

Creed ER (1975) Melanism in 2 spot ladybird - nature and intensity of selection. Proc. R. Soc. Lond. B 190:135-148

Crowson R (1981) The biology of the Coleoptera. Academic Press, London

Dahlsten DL, Zuparko RL, Hajek AE, Rowney DL, Dreistadt SH (1999) Long-term sampling of *Eucallipterus tiliae* (Homoptera: Drepanosiphidae) and associated natural enemies in a northern California site. Environ. Entomol. 28:845-850

Damen WGM, Saridaki T, Averof M (2002) Diverse adaptations of an ancestral gill: A common evolutionary origin for wings, breathing organs, and spinnerets. Curr. Biol. 12:1711-1716

Danchin E, Charmantier A, Champagne FA, Mesoudi A, Pujol B, Blanchet S (2011) Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. Nature Rev. Genet. 12:475-486

Darlington P (1936) Variation and atrophy of flying wings of some carabid beetles (Coleoptera). Ann. Entomol. Soc. Am. 49:136-179

Darwin CR (1859) On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. John Murray, London

Day KR, Docherty M, Leather SR, Kidd NAC (2006) The role of generalist insect predators and pathogens in suppressing green spruce aphid populations through direct mortality and mediation of aphid dropping behavior. Biol. Control 38:233-246

De Clercq P, Bonte M, Van Speybroeck K, Bolckmans K, Deforce K (2005) Development and reproduction of *Adalia bipunctata* (Coleoptera: Coccinellidae) on eggs of *Ephestia kuehniella* (Lepidoptera: Phycitidae) and pollen. Pest Manag. Sci. 61:1129-1132

De Clercq P, Mason PG, Babendreier D (2011) Benefits and risks of exotic biological control agents. BioControl 56:681-698

De Jong PW, Brakefield PM, Geerinck BP (1998) The effect of female mating history on sperm precedence in the two-spot ladybird, *Adalia bipunctata* (Coleoptera, Coccinellidae). Behav. Ecol. 9:559-565

De Jong PW, Gussekloo SWS, Brakefield PM (1996) Differences in thermal balance, body temperature and activity between non-melanic and melanic two-spot ladybird beetles (*Adalia bipunctata*) under controlled conditions. J. Exp. Biol. 199:2655-2666

Dekkers JCM, Hospital F (2002) The use of molecular genetics in the improvement of agricultural populations. Nature Rev. Genet. 3:22-32

Den Boer PJ, Van Huizen THP, Den Boer-Daanje W, Aukema B, Den Bieman CFM (1980) Wing polymorphism and dimorphism in ground beetles as stages in an evolutionary process (Coleoptera, Carabidae). Entomol. Gen. 6:107-134

Desender K (1989) Heritability of wing development and body size in a carabid beetle, *Pogonus chalceus* Marsham, and its evolutionary significance. Oecologia 78:513-520

Dib H, Simon S, Sauphanor B, Capowiez Y (2010) The role of natural enemies on the population dynamics of the rosy apple aphid, *Dysaphis plantaginea* Passerini (Hemiptera: Aphididae) in organic apple orchards in south-eastern France. Biol. Control 55:97-109

Dill LM, Fraser AHG, Roitberg BD (1990) The economics of escape behaviour in the pea aphid, *Acyrthosiphon pisum*. Oecologia 83:473-478

Dixon AFG (1971a) Role of aphids in wood formation. 1. Effect of sycamore aphid, *Drepanosiphum platanoides* (Schr) (Aphididae), on growth of sycamore, *Acer pseudoplatanus* (L). J. Appl. Ecol. 8:165-179

Dixon AFG (1971b) Role of aphids in wood formation. 2. Effect of lime aphid, *Eucallipterus tiliae* L. (Aphididae), on growth of lime, *Tilia* x *vulgaris* Hayne. J. Appl. Ecol. 8:393-399

Dixon AFG (1971c) Role of intra-specific mechanisms and predation in regulating numbers of lime aphid, *Eucallipterus tiliae* L. Oecologia 8:179-193

Dixon AFG (1998) Aphid ecology. Chapman & Hall, London

Dixon AFG (2000) Insect predator-prey dynamics. Ladybird beetles and biological control. Cambridge University Press, Cambridge

Dixon AFG, Agarwala BK (1999) Ladybird-induced life-history changes in aphids. Proc. R. Soc. Lond. B 266:1549-1553

Dixon AFG, Barlow ND (1979) Population regulation in the lime aphid. Zool. J. Linn. Soc. 67:225-237

Dobzhansky T (1924) Die geographische und individuelle Variabilitat von *Harmonia axyridis* Pall in ihren Wechselbeziehungen. Biol. Zentralblatt 44 401–421

Dobzhansky T (1933) Geographical variation in lady-beetles. Am. Nat. 67:97–126

Dolenska M, Nedved O, Vesely P, Tesarova M, Fuchs R (2009) What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: colour, pattern or general look? Biol. J. Linnean Soc. 98:234-242

Doumbia M, Hemptinne JL, Dixon AFG (1998) Assessment of patch quality by ladybirds: role of larval tracks. Oecologia 113:197-202

Dreistadt SH, Dahlsten DL (1988) Tuliptree aphid honeydew management. J. Arboriculture 14:209-214

Duveau F, Felix MA (2012) Role of pleiotropy in the evolution of a cryptic developmental variation in *Caenorhabditis elegans*. PLoS Biology 10

Dworkin I, Kennerly E, Tack D, Hutchinson J, Brown J, Mahaffey J, Gibson G (2009) Genomic consequences of background effects on *scalloped* mutant expressivity in the wing of *Drosophila melanogaster*. Genetics 181:1065-1076

Dybas HS (1978) Polymorphism in featherwing beetles, with a revision of genus *Ptinellodes* (Coleoptera Ptiliidae). Ann. Entomol. Soc. Am. 71:695-714

Eilenberg J, Hajek AE, Lomer C (2001) Suggestions for unifying the terminology for biological control. BioControl:387-400

Engel MS, Grimaldi DA (2004) New light shed on the oldest insect. Nature 427:627-630

Ettifouri M, Ferran A (1993) Influence of larval rearing diet on the intensive searching behavior of *Harmonia axyridis* (Col, Coccinellidae) larvae. Entomophaga 38:51-59

Evans EW (2004) Habitat displacement of North American ladybirds by an introduced species. Ecology 85:637-647

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics Longman Group Ltd Harlow, UK

Ferran A, Giuge L, Tourniaire R, Gambier J, Fournier D (1998) An artificial non-flying mutation to improve the efficiency of the ladybird *Harmonia axyridis* in biological control of aphids. BioControl 43:53-64

Ferran A, Niknam H, Kabiri F, Picart JL, DeHerce C, Brun J, Iperti G, Lapchin L (1996) The use of *Harmonia axyridis* larvae (Coleoptera: Coccinellidae) against *Macrosiphum rosae* (Hemiptera: Sternorrhyncha: Aphididae) on rose bushes. Eur. J. Entomol. 93:59-67

Fonseca MG, Zarbin PHG (2009) Mating behaviour and evidence for sex-specific pheromones in *Hedypathes betulinus* (Coleoptera: Cerambycidae: Lamiinae). J. Appl. Entomol. 133:695-701

Frankino WA, Zwaan BJ, Stern DL, Brakefield PM (2007) Internal and external constraints in the evolution of morphological allometries in a butterfly. Evolution 61:2958-2970

Fukaya M, Akino T, Yasuda T, Yasui H, Wakamura S (2004) Visual and olfactory cues for mate orientation behaviour in male white-spotted longicorn beetle, *Anoplophora malasiaca*. Entomol. Exp. Appl. 111:111-115

Fukaya M, Akino T, Yasui H, Yasuda T, Wakamura S, Yamamura K (2005) Effects of size and color of female models for male mate orientation in the white-spotted longicorn beetle *Anoplophora malasiaca* (Coleoptera: Cerambycidae). Appl. Entomol. Zoolog. 40:513-519

Fukaya M, Yasuda T, Wakamura S, Honda H (1996) Reproductive biology of the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae). 3. Identification of contact sex pheromone on female body surface. J. Chem. Ecol. 22:259-270

Futuyma DJ (2005) Evolution. Sinauer Associates, Sunderland, USA

Geiselhardt S, Otte T, Hilker M (2009) The role of cuticular hydrocarbons in male mating behavior of the mustard leaf beetle, *Phaedon cochleariae* (F.). J. Chem. Ecol. 35:1162-1171

Gibson G, Dworkin I (2004) Uncovering cryptic genetic variation. Nature Rev. Genet. 5:681-690

Gibson G, van Helden S (1997) Is function of the Drosophila homeotic gene *Ultrabithorax* canalized? Genetics 147:1155-1168

Gibson G, Wemple M, van Helden S (1999) Potential variance affecting homeotic *Ultrabithorax* and *Antennapedia* phenotypes in *Drosophila melanogaster*. Genetics 151:1081-1091

Gillespie JH (2001) Is the population size of a species relevant to its evolution? Evolution 55:2161-2169

Gillespie JH (2006) Stochastic processes in evolution. In: Fox CW and Wolf JB (eds) Evolutionary genetics. Concepts and case studies. Oxford University Press, Oxford, pp. 65-79

Ginzel MD (2010) Hydrocarbons as contact pheromones of longhorned beetles (Coleoptera: Cerambycidae). In: Blomquist GJ and Bagnères A (eds) Insect hydrocarbons. Biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, UK, pp. 492

Ginzel MD, Blomquist GJ, Millar JG, Hanks LM (2003) Role of contact pheromones in mate recognition in *Xylotrechus colonus*. J. Chem. Ecol. 29:533-545

Ginzel MD, Hanks LM (2003) Contact pheromones as mate recognition cues of four species of longhorned beetles (Coleoptera: Cerambycidae). J. Ins. Behav. 16:181-187

Glen DM, Barlow ND (1980) Interaction of a population of the black-kneed capsid, *Blepharidopterus angulatus*, and its prey, the lime aphid. Ecol. Entomol. 5:335-344

Golden KL, Meinke LJ, Stanleysamuelson DW (1992) Cuticular hydrocarbon discrimination of *Diabrotica* (Coleoptera, Chrysomelidae) sibling species. Ann. Entomol. Soc. Am. 85:561-570

Gotoh A, Sameshima S, Tsuji K, Matsumoto T, Miura T (2005) Apoptotic wing degeneration and formation of an altruism-regulating glandular appendage (gemma) in the ponerine ant *Diacamma* sp. from Japan (Hymenoptera, Formicidae, Ponerinae). Dev. Genes Evol. 215:69-77

Gould F (2008) Broadening the application of evolutionarily based genetic pest management. Evolution 62:500-510

Grimaldi DA, Engel MS (2005) Evolution of the Insects. Cambridge University Press, Cambridge

Gurney B, Hussey NW (1970) Evaluation of some Coccinellid species for biological control of aphids in protected cropping. Ann. Appl. Biol. 65:451-&

Haddrill PR, Shuker DM, Amos W, Majerus MEN, Mayes S (2008) Female multiple mating in wild and laboratory populations of the two-spot ladybird, *Adalia bipunctata*. Mol. Ecol. 17:3189-3197

Haddrill PR, Shuker DM, Mayes S, Majerus MEN (2007) Temporal effects of multiple mating on components of fitness in the two-spot ladybird, *Adalia bipunctata* (Coleoptera : Coccinellidae). Eur. J. Entomol. 104:393-398

Hajek AE (2004) Natural enemies. An introduction to biological control. Cambridge University Press, Cambridge, UK

Haldane JBS (1919) The combination of linkage values, and the calculation of distances between the loci of linked factors. J. Genet. 8:299–309

Hämäläinen M (1976) Rearing the univoltine ladybeetles, *Coccinella septempunctata* and *Adalia bipunctata* (Col., Coccinellidae), all year round in the laboratory. A.. Agric. Fenn. 15:66-71

Hämäläinen M (1977) Control of aphids on sweet peppers, chrysantemums and roses in small greenhouses using ladybeetles *Coccinella septempunctata* and *Adalia bipunctata* (Col, Coccinellidae). A.. Agric. Fenn. 16:117-131

Hämäläinen M (1980) Evaluation of two native coccinellids for aphid control in glasshouses IOBC/WPRS Bulletin 59-63

Hämäläinen M, Markkula M (1977) Cool storage of *Coccinella septempunctata* and *Adalia bipunctata* (COL, Coccinellidae) eggs for use in biological control in greenhouses. A.. Agric. Fenn. 16:132-136

Hammond PM (1985) Dimorphism of wings, wing-folding and wing-toiletry devices in the ladybird, *Rhyzobius litura* (F) (Coleoptera, Coccinellidae), with a discussion of inter-population variation in this and other wing-dimorphic beetle species. Biol. J. Linnean Soc. 24:15-33

Hayden EJ, Ferrada E, Wagner A (2011) Cryptic genetic variation promotes rapid evolutionary adaptation in an RNA enzyme. Nature 474:92-U120

Heimbach U (1982) Zur Honigtauproduktion von zwei Callaphididen an Linde und Eiche. Apidologie 13:331-332

Held LI (2002) Imaginal discs. The genetic and cellular logic of pattern formation. Cambridge University Press, Cambridge

Hemptinne JL, Desprets A (1986) Pollen as a spring food for *Adalia bipunctata* L. In: Hodek I (ed) Ecology of Aphidophaga. Academia and W. Junk, Prague and Dordrecht pp. 29-35

Hemptinne JL, Dixon AFG, Adam B (2001) Do males and females of the two-spot ladybird, *Adalia bipunctata* (L.), differ in when they mature sexually? J. Ins. Behav. 14:411-419

Hemptinne JL, Dixon AFG, Coffin J (1992) Attack strategy of ladybird beetles (Coccinellidae) - factors shaping their numerical response. Oecologia 90:238-245

Hemptinne JL, Dixon AFG, Lognay G (1996) Searching behaviour and mate recognition by males of the two-spot ladybird beetle, *Adalia bipunctata*. Ecol. Entomol. 21:165-170

Hemptinne JL, Lognay G, Dixon AFG (1998) Mate recognition in the two-spot ladybird beetle, *Adalia bipunctata*: role of chemical and behavioural cues. J. Ins. Phys. 44:1163-1171

Hemptinne JL, Naisse J (1987) Ecophysiology of the reproductive activity of *Adalia bipunctata* L. Med.Fac. Landbouw., Rijksuniv. Gent 52:225-233

Hermisson J, Wagner GP (2004) The population genetic theory of hidden variation and genetic robustness. Genetics 168:2271-2284

Hiltpold I, Baroni M, Toepfer S, Kuhlmann U, Turlings TCJ (2010) Selection of entomopathogenic nematodes for enhanced responsiveness to a volatile root signal helps to control a major root pest. J. Exp. Biol. 213:2417-2423

Hodek I (1973) Biology of Coccinellidae. Dr. W. Junk N.V., The Hague

Hodek I, Evans EW (2012) Food relationships. In: Hodek I, Van Emden HF and Honěk A (eds) Ecology and behaviour of the ladybird beetles (Coccinellidae). Blackwell Publishing Ltd, Chichester, pp. 141-274

Hodek I, Honěk A (1996) Ecology of Coccinellidae. Kluwer Academic Publishers, Dordrecht/Boston/London

Hodek I, Iperti G, Hodková M (1993) Long-distance flights in Coccinellidae (Coleoptera). Eur. J. Entomol. 90:403-414

Hodek I, Van Emden HF, Honěk A (2012) Ecology and behaviour of the ladybird beetles (Coccinellidae). Blackwell Publishing Ltd., Chichester

Hoekstra RF (2003) Adaptive recovery after fitness reduction: the role of population size. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 89-92

Hoffmann MP, Orfanedes MS, Pedersen LH, Kirkwyland JJ, Hoebeke ER, Ayyappath R (1997) Survey of lady beetles (Coleoptera: Coccinellidae) in sweet corn using yellow sticky cards. J. Entomol. Sci. 32:358-369

Honěk A (1985) Habitat preferences of aphidophagous coccinellids (Coleoptera). Entomophaga 30:253-264

Honěk A (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos 66:483-492

Hopper KR, Roush RT, Powell W (1993) Management of genetics of biological-control introductions. Annu. Rev. Entomol. 38:27-51

Howard RW, Blomquist GJ (1982) Chemical ecology and biochemistry of insect hydrocarbons Annu. Rev. Entomol. 27:149-172

Howard RW, Blomquist GJ (2005) Ecological, behavioral, andbiochemical aspects of insect hydrocarbons. Annu. Rev. Entomol. 50:371-393

Hoy MA (1985) Recent advantages in genetics and genetic-improvement of the Phytoseiidae. Annu. Rev. Entomol. 30:345-370

Hoy MA (1986) Use of genetic improvement in biological control. Agric. Ecosyst. Environ. 15:109-119

Hoy MA (2002) Genetic improvement of biocontrol agents. In: Pimentel D (ed) Encyclopedia of pest management. Marcel Dekker, Inc., New York, pp. 329-332

Hoy MA, Cave FE (1986) Screening for thelytocia in the parahaploid Phytoseiid, *Metaseiulus occidentalis* (Nesbitt). Exp. Appl. Acarol. 2:273-276

Hundertmark A (1935) Die Entwicklung der Flügel des Mehlkäfers *Tenebrio molitor*, mit besonderer Berücksichtigung der Häuntungsvorgänge Z. Morphol. Okol. Tiere 30:506-543

Hurst GDD, Sharpe RG, Broomfield AH, Walker LE, Majerus TMO, Zakharov IA, Majerus MEN (1995) Sexually-transmitted disease in a promiscuous insect, *Adalia bipunctata*. Ecol. Entomol. 20:230-236

Ignoffo CM, Garcia C, Dickerson WA, Schmidt GT, Biever KD (1977) Imprisonment of entomophages to increase effectiveness - evaluation of a concept. J. Econ. Entomol. 70:292-294

Iguchi M, Fukushima F, Miura K (2012) Control of *Aphis gossypii* and *Myzus persicae* (Hemiptera: Aphididae) by a flightless strain of *Harmonia axyridis* (Coleoptera: Coccinellidae) on green pepper plants in open fields. Entomol. Sci. 15:127-132

Ikeda H, Nishikawa M, Sota T (2012) Loss of flight promotes beetle diversification. Nat. Commun. 3

Irvin NA, Hoddle MS (2006) The effect of intraspecific competition on progeny sex ratio in *Gonatocerus* spp. for *Homalodisca coagulata* egg masses: Economic implications for mass rearing and biological control. Biol. Control 39:162-170

Ives AR, Kareiva P, Perry R (1993) Response of a predator to variation in prey density at three hierarchical scales: lady beetles feeding on aphids. Ecology 74:1929-1938

Jack J, DeLotto Y (1992) Effect of wing scalloping mutations on *cut* expression and sense organ differentiation in the *Drosophila* wing margin. Genetics 131:353-363

Jackson DJ (1928) The inheritance of long and short wings in the weevil, *Sitona hispidula*, with a discussion of wing reduction among beetles. Trans. Roy. Soc. Edinburgh:665-735

Jalali MA, Michaud JP (2012) Aphid-plant interactions affect the suitability of *Myzus* spp. as prey for the two spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae). Eur. J. Entomol. 109:345-352

Jalali MA, Tirry L, Arbab A, De Clercq P (2010) Temperature-dependent development of the two-spotted ladybeetle, *Adalia bipunctata*, on the green peach aphid, *Myzus persicae*, and a factitious food under constant temperatures. J. Ins. Sci. 10

Jalali MA, Tirry L, De Clercq P (2009) Food consumption and immature growth of *Adalia bipunctata* (Coleoptera: Coccinellidae) on a natural prey and a factitious food. Eur. J. Entomol. 106:193-198

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. Biol. Rev. Cambridge Philosophic. Soc. 75:21-64

Jermy T, Butt BA (1991) Method for screening female sex-pheromone extracts of the Colorado potato beetle. Entomol. Exp. Appl. 59:75-78

Jetter K, Paine TD (2004) Consumer preferences and willingness to pay for biological control in the urban landscape. Biol. Control 30:312-322

Johannes F, Colot V, Jansen RC (2008) Epigenome dynamics: a quantitative genetics perspective. Nature Rev. Genet. 9:883-890

Johnson MW, Tabashnik WJ (1993) Laboratory selection for pesticide resistance in natural enemies. In: Narang SK, Bartlett AC and Faust RM (eds) Applications of genetics to arthropods of biological control significance. CRC Press, Inc., Boca Raton, Florida, pp. 91-105

Kalushkov P (1998) Ten aphid species (Sternorrhyncha : Aphididae) as prey for *Adalia bipunctata* (Coleoptera : Coccinellidae). Eur. J. Entomol. 95:343-349

Kearns PWE, Tomlinson IPM, Odonald P, Veltman CJ (1990) Non-random mating in the two-spot ladybird (*Adalia bipunctata*): I. A reassessment of the evidence. Heredity 65:229-240

Kehrli P, Wyss E (2001) Effects of augmentative releases of the coccinellid, *Adalia bipunctata*, and of insecticide treatments in autumn on the spring population of aphids of the genus *Dysaphis* in apple orchards. Entomol. Exp. Appl. 99:245-252

Kieckhefer RW, Olson GA (1974) Dispersal of marked adult coccinellids from crops in South-Dakota. J. Econ. Entomol. 67:52-54

Kindlmann P, Ameixa O, Dixon AFG (2011) Ecological effects of invasive alien species on native communities, with particular emphasis on the interactions between aphids and ladybirds. BioControl 56:469-476

Kitami T, Nadeau JH (2002) Biochemical networking contributes more to genetic buffering in human and mouse metabolic pathways than does gene duplication. Nature Genet. 32:191-194

Knell RJ, Webberley KM (2004) Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. Biol. Rev. Cambridge Philosophic. Soc. 79:557-581

Koch RL (2003) The multicolored Asian lady beetle *Harmonia axyridis*. A review of its biology, uses in biological control, and non-target impacts. J. Ins. Sci. 3:1-16

Koonin EV (2009) Darwinian evolution in the light of genomics. Nucleic Acids Res. 37:1011-1034

Kuroda T, Miura K (2003) Comparison of the effectiveness of two methods for releasing *Harmonia axyridis* (Pallas) (Coleoptera : Coccinellidae) against *Aphis gossypii* Glover (Homoptera : Aphididae) on cucumbers in a greenhouse. Appl. Entomol. Zoolog. 38:271-274

Kuwayama H, Yaginuma T, Yamashita O, Niimi T (2006) Germ-line transformation and RNAi of the ladybird beetle, *Harmonia axyridis*. Ins. Mol. Biol. 15:507-512

Laland KN, Sterelny K, Odling-Smee J, Hoppitt W, Uller T (2011) Cause and effect in biology revisited: Is Mayr's proximate-ultimate dichotomy still useful? Science 334:1512-1516

Le Rouzic A, Carlborg O (2008) Evolutionary potential of hidden genetic variation. Trends Ecol. Evol. 23:33-37

Lelito JP, Domingue MJ, Fraser I, Mastro VC, Tumlinson JH, Baker TC (2011) Field investigation of mating behaviour of *Agrilus cyanescens* and *Agrilus subcinctus*. Can. Entomol. 143:370-379

Lelito JP, Fraser I, Mastro VC, Tumlinson JH, Boroczky K, Baker TC (2007) Visually mediated 'paratrooper copulations' in the mating behavior of *Agrilus planipennis* (Coleoptera : Buprestidae), a highly destructive invasive pest of North American ash trees. J. Ins. Behav. 20:537-552

Leppla NC (2003) Aspects of total quality control for the production of natural enemies. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 19-24

Lindsley DL, Zimm GG (1992) The genome of *Drosophila melanogaster*. Academic Press, London

Lobbia S, Niitsu S, Fujiwara H (2003) Female-specific wing degeneration caused by ecdysteroid in the Tussock Moth, *Orgyia recens*: hormonal and developmental regulation of sexual dimorphism. J. Ins. Sci. 3:11

Lommen STE, De Jong PW, Brakefield PM (2005) Phenotypic plasticity of elytron length in wingless two-spot ladybird beetles, *Adalia bipunctata* (Coleoptera: Coccinellidae). Eur. J. Entomol. 102:553-556

Lommen STE, De Jong PW, Koops KG, Brakefield PM (2012) Genetic linkage between melanism and winglessness in the ladybird beetle *Adalia bipunctata*. Genetica 140: 229–233 [CHAPTER 4]

Lommen STE, Holness TC, Van Kuik AJ, De Jong PW, Brakefield PM (2013) Releases of a natural flightless strain of the ladybird beetle *Adalia bipunctata* reduce aphid-born honeydew beneath urban lime trees. BioControl 58: 195-204 [CHAPTER 3]

Lommen STE, Middendorp CW, Luijten CA, Van Schelt J, Brakefield PM, De Jong PW (2008) Natural flightless morphs of the ladybird beetle *Adalia bipunctata* improve biological control of aphids on single plants. Biol. Control 47:340-346 [CHAPTER 2]

Lommen STE, Saenko SV, Tomoyasu Y, Brakefield PM (2009) Development of a wingless morph in the ladybird beetle, *Adalia bipunctata*. Evol. Dev. 11:278-289 [CHAPTER 6]

Lorenz MW (2007) Oogenesis-flight syndrome in crickets: Age-dependent egg production, flight performance, and biochemical composition of the flight muscles in adult female *Gryllus bimaculatus*. J. Ins. Phys. 53:819-832

Losey JE, Denno RF (1998) The escape response of pea aphids to foliar-foraging predators: factors affecting dropping behaviour. Ecol. Entomol. 23:53-61

Lozier JD, Roderick GK, Mills NJ (2008) Evolutionarily significant units in natural enemies: Identifying regional populations of *Aphidius transcaspicus* (Hymenoptera : Braconidae) for use in biological control of mealy plum aphid. Biol. Control 46:532-541

Luo SL, Zhuge PP, Wang MQ (2011) Mating behavior and contact pheromones of *Batocera horsfieldi* (Hope) (Coleoptera: Cerambycidae). Entomol. Sci. 14:359-363

Lusis JJ (1961) On the biological meaning of colour polymorphism of lady-beetle *Adalia bipunctata* L. Latvijas Entomologs 4:3-29

Lynch M (2007) The frailty of adaptive hypotheses for the origins of organismal complexity. Proc. Natl. Acad. Sci. U. S. A. 104:8597-8604

Mackauer M (1976) Genetic problems in production of biological-control agents. Annu. Rev. Entomol. 21:369-385

Majerus M, Odonald P, Weir J (1982) Evidence for preferential mating in *Adalia bipunctata.* Heredity 49:37-49

Majerus MEN (1994) Ladybirds. Collins, London

Majerus MEN (1998) Melanism : evolution in action. Oxford University Press, Oxford [etc.]

Majerus MEN, Kearns PWE (1989) Ladybirds. Richmond Publishing Company, Slough

Majerus MEN, Odonald P, Kearns PWE, Ireland H (1986) Genetics and evolution of female choice. Nature 321:164-167

Majerus MEN, Strawson V, Roy HE (2006) The potential impacts of the arrival of the harlequin ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), in Britain. Ecol. Entomol. 31:207-215

Majerus MEN, Zakharov IA (2000) Does thermal melanism maintain melanic polymorphism in the two-spot ladybird Adalia bipunctata. (Coleoptera: Coccinellidae)? Zhurnal Obshchei Biologii 61:381-392

Maki H (2002) Origins of spontaneous mutations: Specificity and directionality of base-substitution, frameshift, and sequence-substitution mutageneses. Annu. Rev. Genet. 36:279-303

Mally CW (1916) On the selection and breeding of desirable strains of beneficial insects. S. Afr. J. Sci.:369-385

Mann JA, Tatchell GM, Dupuch MJ, Harrington R, Clark SJ, McCartney HA (1995) Movement of apterous *Sitobion avenae* (Homoptera, Aphididae) in response to leaf disturbances caused by wind and rain. Ann. Appl. Biol. 126:417-427

Marples NM, Brakefield PM, Cowie RJ (1989) Differences between the seven-spot and two-spot ladybird beetles (Coccinellidae) in their toxic effects on a bird predator. Ecol. Entomol. 14:79-84

Marples NM, De Jong PW, Ottenheim MM, Verhoog MD, Brakefield PM (1993) The inheritance of a wingless character in the 2-spot ladybird (*Adalia bipunctata*). Entomol. Exp. Appl. 69:69-73

Marples NM, Van Veelen W, Brakefield PM (1994) The relative importance of color, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. Anim. Behav. 48:967-974

Martini X, Haccou P, Olivieri I, Hemptinne JL (2009) Evolution of cannibalism and female's response to oviposition-deterring pheromone in aphidophagous predators. J. Anim. Ecol. 78:964-972

Masel J (2006) Cryptic genetic variation is enriched for potential adaptations. Genetics 172:1985-1991

Mawson CA (1948) Honeydew on lime trees. Nature 161:176-176

Mayr E (1961) Cause and effect in biology - kinds of causes, predictability, and teleology are viewed by a practicing biologist. Science 134:1501-1506

Mayr E (2002) What evolution is. Basic Books, New York

McGuigan K, Sgro CM (2009) Evolutionary consequences of cryptic genetic variation. Trends Ecol. Evol. 24:305-311

McLellan ID (1999) A revision of *Zelandoperla* Tillyard (Plecoptera: Gripopterygidae: Zelandoperlinae). N. Z. J. Zool. 26:199-219

Michaud JP (2012) Coccinellids in biological control. In: Hodek I, Van Emden HF and Honěk A (eds) Ecology and behaviour of the ladybird beetles (Coccinellidae). Blackwell Publishing Ltd, Chichester, pp. 488-519

Michie LJ, Mallard F, Majerus MEN, Jiggins FM (2010) Melanic through nature or nurture: genetic polymorphism and phenotypic plasticity in *Harmonia axyridis*. J. Evol. Biol. 23:1699-1707

Michie LJ, Masson A, Ware RL, Jiggins FM (2011) Seasonal phenotypic plasticity: wild ladybirds are darker at cold temperatures. Evol. Ecol. 25:1259-1268

Milloz J, Duveau F, Nuez I, Felix MA (2008) Intraspecific evolution of the intercellular signaling network underlying a robust developmental system. Genes Dev. 22:3064-3075

Mills NJ (1979) *Adalia bipunctata* (L.) as a generalist predator of aphids. University East Anglia, Norwich,

Mills NJ (1981) Essential and alternative foods for some British Coccinellidae. Entomol. Gaz. 32:197-202

Mills NJ (1982) Voracity, cannibalism and coccinellid predation. Ann. Appl. Biol. 101:144-148

Ming QL, Lewis SM (2010) Mate recognition and sex differences in cuticular hydrocarbons of the diurnal firefly *Ellychnia corrusca* (Coleoptera: Lampyridae). Ann. Entomol. Soc. Am. 103:128-133

Minks AK, Harrewijn P (1987) Aphids, their biology, natural enemies and control. Elsevier, Amsterdam

Minoretti N, Weisser WW (2000) The impact of individual ladybirds (*Coccinella septempunctata*, Coleoptera: Coccinellidae) cell aphid colonies. Eur. J. Entomol. 97:475-479

Mole S, Zera AJ (1993) Differential allocation of resources underlies the dispersal-reproduction trade-off in the wing-dimorphic cricket, *Gryllus rubens*. Oecologia 93:121-127

Morjan CL, Rieseberg LH (2004) How species evolve collectively: implications of gene flow and selection for the spread of advantageous alleles. Mol. Ecol. 13:1341-1356

Mutis A, Parra L, Palma R, Pardo F, Perich F, Quiroz A (2009) Evidence of contact pheromone use in mating behavior of the raspberry weevil (Coleoptera: Curculionidae). Environ. Entomol. 38:192-197

Nachappa P, Margolies DC, Nechols JR, Morgan TJ (2010) Response of a complex foraging phenotype to artificial selection on its component traits. Evol. Ecol. 24:631-655

Nadeau JH (2001) Modifier genes in mice and humans. Nature Rev. Genet. 2:165-174

Nahmad M, Glass L, Abouheif E (2008) The dynamics of developmental system drift in the gene network underlying wing polyphenism in ants: a mathematical model. Evol. Dev. 10:360-374

Narang SK, Tabashnik WJ, Faust RM (1993) Complexities of population genetic structure and implications for biological control programs. In: Narang SK, Bartlett AC and Faust RM (eds) Applications of genetics to arthropods of biological control significance. CRC Press, Inc., Boca Raton, Florida, pp. 19-52

Nelson EH, Matthews CE, Rosenheim JA (2004) Predators reduce prey population growth by inducing changes in prey behavior. Ecol. Lett. 85:1853-1858

Ng M, Diaz-Benjumea FJ, Cohen SM (1995) *nubbin* encodes a POU-domain protein required for proximal-distal patterning in the *Drosophila* wing. Development 121:589-599

Ng M, Diaz-Benjumea FJ, Vincent J, Wu J, Cohen SM (1996) Specification of the wing by localized expression of wingless protein. Nat. Commun. 381:316-318

Niimi T, Kuwayama H, Yaginuma T (2005) Larval RNAi applied to the analysis of postembryonic development in the ladybird beetle, *Harmonia axyridis*. J. Insect Biotechnol. Sericology 74:95-102

Niitsu S (2001) Wing degeneration due to apoptosis in the female of the winter moth, *Nyssiodes lefuarius*. Entomol. Sci. 4:1-7

Niitsu S (2003) Postembryonic development of the wing imaginal discs in the female wingless bagworm moth *Eumeta variegata* (Lepidoptera, Psychidae). J. Morphol. 257:164-170

Nunney L (2003) Managing captive populations for release: a population-genetic perspective. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 73-88

Obrycki JJ, Harwood JD, Kring TJ, O'Neil RJ (2009) Aphidophagy by Coccinellidae: Application of biological control in agroecosystems. Biol. Control 51:244-254

Obrycki JJ, Kring TJ (1998) Predaceous Coccinellidae in biological control. Annu. Rev. Entomol. 43:295-321

Ode PJ, Hardy ICW (2008) Parasitoid sex ratios and biological control. In: Wajnberg E, Bernstein C and Van Alphen J (eds) Behavioral ecology of insect parasitoids. From theoretical approaches to field applications. Blackwell Publishing, Malden, Massachusetts, pp. 253-291

Ohde T, Masumoto M, Morita-Miwa M, Matsuura H, Yoshioka H, Yaginuma T, Niimi T (2009) *Vestigial* and *scalloped* in the ladybird beetle: a conserved function in wing development and a novel function in pupal ecdysis. Ins. Mol. Biol. 18:571-581

Olszak RW (1986) The effectiveness of three species of coccinellid adults in controlling small colonies of the green apple aphid (*Aphis pomi*). In: Hodek I (ed) Ecology of Aphidophaga. Academia/Junk, Prague/Dordrecht, pp. 381-384

Omkar, Pervez A (2005) Ecology of two-spotted ladybird, *Adalia bipunctata*: a review. J. Appl. Entomol. 129:465-474

Panganiban G, Sebring A, Nagy L, Carroll SB (1995) The development of crustacean limbs and the evolution of arthropods. Science 270:1363-1366

Patel NH, Kornberg TB, Goodman CS (1989) Expression of *engrailed* during segmentation in grasshopper and crayfish. Development 107:201-212

Perry JC, Rowe L (2008) Neither mating rate nor spermatophore feeding influences longevity in a ladybird beetle. Ethology 114:504-511

Perry JC, Sharpe DMT, Rowe L (2009) Condition-dependent female remating resistance generates sexual selection on male size in a ladybird beetle. Anim. Behav. 77:743-748

Peschke K (1978) Female sex-pheromone of a Staphylinid beetle, *Aleochara curtula*. J. Ins. Phys. 24:197-200

Peschke K (1979) Tactile orientation by mating males of the staphilinid beetle, *Aleochara curtula*, relative to the setal fields of the female. Phys. Entomol. 4:155-159

Peschke K (1987) Cuticular hydrocarbons regulate mate recognition, male aggression, and female choice of the rove beetle, *Aleochara curtula*. J. Chem. Ecol. 13:1993-2008

Peterson MA, Dobler S, Larson EL, Juarez D, Schlarbaum T, Monsen KJ, Francke W (2007) Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation between hybridising *Chrysochus* (Coleoptera : Chrysomelidae). Chemoecology 17:87-96

Polaczyk PJ, Gasperini R, Gibson G (1998) Naturally occurring genetic variation affects *Drosophila* photoreceptor determination. Dev. Genes Evol. 207:462-470

Pope RD (1977) Brachyptery and wing polymorphism among Coccinellidae (Coleoptera). Syst. Entomol. 2:59-66

Powell W, Pell JK (2007) Biological Control. In: Van Emden HF and Harrington R (eds) Aphids as crop pests. CAB International, pp. 469-513

Pureswaran DS, Poland TM (2009) The role of olfactory cues in short-range mate finding by the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). J. Ins. Behav. 22:205-216

Quennedey A, Quennedey B (1990) Morphogenesis of the Wing Anlagen in the mealworm beetle *Tenebrio molitor* during the last larval instar. Tissue Cell 22:721-740

Quennedey A, Quennedey B (1999) Development of the wing discs of *Zophobas atratus* under natural and experimental conditions: occurrence of a gradual larval-pupal commitment in the epidermis of tenebrionid beetles. Cell Tissue Res. 296:619-634

R Development Core Team (2007) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Ribaut JM, Hoisington D (1998) Marker-assisted selection: new tools and strategies. Trends Plant Sci. 3:236-239

Roff DA (1986) The evolution of wing dimorphism in insects. Evolution 40:1009-1020

Roff DA (1990) The evolution of flightlessness in insects. Ecol. Monogr. 60:389-421

Roff DA (1994a) The evolution of flightlessness: is history important? Evol. Ecol. 8:639-657

Roff DA (1994b) Why is there so much genetic variation for wing dimorphism? Res. Popul. Ecol. 36:145-150

Roff DA, Fairbairn DJ (1991) Wing dimorphisms and the evolution of migratory polymorphisms among the Insecta. Am. Zool. 31:243-251

Roff DA, Mostowy S, Fairbairn DJ (2002) The evolution of trade-offs: Testing predictions on response to selection and environmental variation. Evolution 56:84-95

Rosenheim JA, Hoy MA (1988) Genetic-improvement of a parasitoid biological-control agent - artificial selection for insecticide resistance in *Aphytis melinus* (Hymenoptera, Aphelinidae). J. Econ. Entomol. 81:1539-1550

Roy HE, Wajnberg E (2008) From biological control to invasion: the ladybird *Harmonia axyridis* as a model species. BioControl 53:1-4

Rutherford SL (2000) From genotype to phenotype: buffering mechanisms and the storage of genetic information. BioEssays 22:1095-1105

Rutherford SL (2003) Between genotype and phenotype: Protein chaperones and evolvability. Nature Rev. Genet. 4:263-274

Rutherford SL, Lindquist S (1998) Hsp90 as a capacitor for morphological evolution. Nature 396:336-342

Salame L, Glazer I, Chubinishvilli MT, Chkhubianishvili T (2010) Genetic improvement of the desiccation tolerance and host-seeking ability of the entomopathogenic nematode *Steinernema feltiae*. Phytoparasitica 38:359-368

Sameshima S, Miura T, Matsumoto T (2004) Wing disc development during caste differentiation in the ant *Pheidole megacephala* (Hymenoptera: Formicidae). Evol. Dev. 6:336-341

Sangster TA, Salathia N, Lee HN, Watanabe E, Schellenberg K, Morneau K, Wang H, Undurraga S, Queitsch C, Lindquist S (2008a) HSP90-buffered genetic variation is common in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U. S. A. 105:2969-2974

Sangster TA, Salathia N, Undurraga S, Milo R, Schelienberg K, Lindquist S, Queitsch C (2008b) HSP90 affects the expression of genetic variation and developmental stability in quantitative traits. Proc. Natl. Acad. Sci. U. S. A. 105:2963-2968

Scali V, Creed ER (1975) Influence of climate on melanism in two-spot ladybird, *Adalia bipunctata*, in central Italy. Trans. Roy. Entomol. Soc. Lond. 127:163-169

Schlichting CD (2008) Hidden reaction norms, cryptic genetic variation, and evolvability. Ann. N. Y. Acad. Sci. 1133:187-203

Secretariat of the International Plant Protection Convention, FAO (2005) Guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms. Food and Agriculture Organization of the United Nations, Rome

Seko T, Yamashita K, Miura K (2008) Residence period of a flightless strain of the ladybird beetle *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) in open fields. Biol. Control 47:194-198

Shapiro DI, Glazer I, Segal D (1997) Genetic improvement of heat tolerance in *Heterorhabditis bacteriophora* through hybridization. Biol. Control 8:153-159

Smith DS (1964) Structure and development of flightless Coleoptera - light and electron microscopic study of wings, thoracic exoskeleton, and rudimentary flight musculature. J. Morphol. 114:107-183

Smith SG (1953) Chromosome numbers of Coleoptera. Heredity 7:31-48

Sokal RR, Rohlf FJ (1995) Biometry: The principles and practice of statistics in biological research. W.H. Freeman, New York

Sollars V, Lu XY, Xiao L, Wang XY, Garfinkel MD, Ruden DM (2003) Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. Nature Genet. 33:70-74

Spofford JB (1956) The relation between expressivity and selection against eyeless in *Drosophila melanogaster*. Genetics 41:938-959

Steiger S, Peschke K, Francke W, Muller JK (2007) The smell of parents: breeding status influences cuticular hydrocarbon pattern in the burying beetle *Nicrophorus vespilloides*. Proc. R. Soc. B 274:2211-2220

Steiger S, Whitlow S, Peschke K, Muller JK (2009) Surface chemicals inform about sex and breeding status in the biparental burying beetle *Nicrophorus vespilloides*. Ethology 115:178-185

Strauch O, Oestergaard J, Hollmer S, Ehlers RU (2004) Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding. Biol. Control 31:218-226

Sugeno W, Hori M, Matsuda K (2006) Identification of the contact sex pheromone of *Gastrophysa atrocyanea* (Coleoptera : Chrysomelidae). Appl. Entomol. Zoolog. 41:269-276

Tabone E, Bardon C, Desneux N, Wajnberg E (2010) Parasitism of different *Trichogramma* species and strains on *Plutella xylostella* L. on greenhouse cauliflower. Journal of Pest Science 83:251-256

Tanigaki T, Yamaoka R, Sota T (2007) The role of cuticular hydrocarbons in mating and conspecific recognition in the closely related longicorn beetles *Pidonia grallatrix* and *P. takechii*. Zool. Sci. 24:39-45

Tauber CA, Tauber MJ, Giffin JG (2007) Flightless Hawaiian Hemerobiidae (Neuroptera): Comparative morphology and biology of a brachypterous species, its macropterous relative and intermediate forms. Eur. J. Entomol. 104:787-800

Tautz D (1992) Redundancies, development and the flow of information. BioEssays 14:263-266

Thayer MK (1992) Discovery of sexual wing dimorphism in Staphylinidae (Coleoptera) - *Omalium flavidum*, and a discussion of wing dimorphism in insects. Journal of the New York Entomological Society 100:540-573

Tian DC, Wang Q, Zhang PF, Araki H, Yang SH, Kreitman M, Nagylaki T, Hudson R, Bergelson J, Chen JQ (2008) Single-nucleotide mutation rate increases close to insertions/deletions in eukaryotes. Nature 455:105-108

Timofeeff-Ressovsky NW (1940) Zur Analyse des Polymorphismus bei *Adalia bipunctata* L. Biol. Zentralblatt 60:130-137

Tomanovic Z, Kavallieratos NG, Stary P, Petrovic-Obradovic O, Athanassiou CG, Stanisavljevic LZ (2008) Cereal aphids (Hemiptera : Aphidoidea) in Serbia: Seasonal dynamics and natural enemies. Eur. J. Entomol. 105:495-501

Tomlinson IPM, Kearns PWE, Veltman CJ (1995) Nonrandom mating in the 2-spot ladybird (*Adalia bipunctata*) - the influence of weight on mating success. Behavior Genetics 25:467-474

Tomoyasu Y, Denell RE (2004) Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Dev. Genes Evol. 214:575-578

Tomoyasu Y, Wheeler SR, E. DR (2005) *Ultrabithorax* is required for membranous wing identity in the beetle *Tribolium castaneum*. Nature 433:643-647

Tourniaire R, Ferran A, Gambier J, Giuge L, Bouffault F (1999) Locomotor behavior of flightless *Harmonia axyridis* Pallas (Col., Coccinellidae). J. Ins. Behav. 12:545-558

Tourniaire R, Ferran A, Giuge L, Piotte C, Gambier J (2000) A natural flightless mutation in the ladybird, *Harmonia axyridis*. Entomol. Exp. Appl. 96:33-38

Tower WL (1903) The origin and development of the wings of Coleoptera. Zool. Jahr. Abt. Anat. Ontog. Thiere:517-572

Tribolium Genome Sequence Consortium (2008) The genome of the model beetle and pest *Tribolium castaneum*. Nature 452:949-955

Trouvé C, Ledee S, Ferran A, Brun J (1997) Biological control of the damson-hop aphid, *Phorodon humuli* (Hom.: Aphididae), using the ladybeetle *Harmonia axyridis* (Col.: Coccinellidae). Entomophaga 42:57-62

True JR (2003) Insect melanism: the molecules matter. Trends Ecol. Evol. 18:640-647

Ueno H, de Jong PW, Brakefield PM (2004) Genetic basis and fitness consequences of winglessness in the two-spot ladybird beetle, *Adalia bipunctata*. Heredity 93:283-289

Van 't Hof AE, Edmonds N, Dalikova M, Marec F, Saccheri IJ (2011) Industrial melanism in British peppered moths has a singular and recent mutational origin. Science 332:958-960

Van 't Hof AE, Saccheri IJ (2010) Industrial melanism in the peppered moth is not associated with genetic variation in canonical melanisation gene candidates. Plos One 5

Van Driesche R, Hoddle M, Center T (2008) Control of pests and weeds by natural enemies: an introduction to biological control. Blackwell Publishing

Van Houten YM, Van Stratum P, Bruin J, Veerman A (1995) Selection for non-diapause in *Amblyseius cucumeris* and *Amblyseius barkeri* and exploration of the effectiveness of selected strains for thrips control. Entomol. Exp. Appl. 77:289-295

Van Lenteren JC (1988) Evaluating the effectiveness of natural enemies. In: Niemczyk E and Dixon AFG (eds) Ecology and Effectiveness of Aphidophaga. SPB Academic Publishing, Den Haag, pp. 175–181

Van Lenteren JC (2003a) Commercial availability of biological control agents. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 167-180

Van Lenteren JC (2003b) Need for quality control of mass-produced biological control agents. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 1-18

Van Lenteren JC (2003c) Quality control and production of biological control agents. Theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 327

Van Lenteren JC (2012) The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. BioControl 57:1-20

Van Lenteren JC, Cock MJW, Brodeur J, Barratt BIP, Bigler F, Bolckmans K, Haas F, Mason PG, Parra JRP (2011) Will the Convention on Biological Diversity put an end to biological control? Rev. Bras. Entomol. 55:1-5

Van Lenteren JC, Hale A, Klapwijk JN, Van Schelt J, Steinberg S (2003) Guidelines for quality control of commercially produced natural enemies. In: Van Lenteren JC (ed) Quality control and production of biological control agents theory and testing procedures. CABI Publishing, Wallingford, UK, pp. 265-304

Van Lenteren JC, Loomans AJM, Babendreier D, Bigler F (2008) *Harmonia axyridis*: an environmental risk assessment for Northwest Europe. BioControl 53:37-54

Vink CJ, Barratt BIP, Phillips CB, Barton DM (2012) Moroccan specimens of *Microctonus aethiopoides* spice our understanding of genetic variation in this internationally important braconid parasitoid of adult weevils. BioControl 57:751-758

Vogler AP, Timmermans M (2012) Speciation: don't fly and diversify? Curr. Biol. 22:R284-R286

Waddington CH (1957) The stratgey of the genes. Allen and Unwin, London

Wagner DL, Liebherr JK (1992) Flightlessness in insects. Trends Ecol. Evol. 7:216-220

Wajnberg E (1991) Quality control of mass-reared arthropods: A genetical and statistical approach. In: Bigler F (ed) Proceedings of the 5th Workshop on Quality Control of mass-reared arthropods, Wageningen, Netherlands, pp. 15-25

Wajnberg E (2004) Measuring genetic variation in natural enemies used for biological control: Why and how? In: Ehler L, Sforza R and Mateille T (eds) Genetics, Evolution and Biological Control. CAB International, Wallingford, pp. 19-37

Wajnberg E (2010) Genetics of the behavioral ecology of egg parasitoids. In: Consoli FL, Parra JRP and Zucchi RA (eds) Egg parasitoids in agroecosystems with emphasis on *Trichogramma*. Springer Dordrecht, pp. 149-156

Wajnberg E, Curty C, Jervis M (2012) Intra-population genetic variation in the temporal pattern of egg maturation in a parasitoid wasp. Plos One 7

Ward JP (1981) Mating behaviour and the mechanism of male orientation in the anobiid bread beetle, *Stegobium paniceum*. Phys. Entomol. 6:213-217

Webberley KM, Hurst GDD, Buszko J, Majerus MEN (2002) Lack of parasite-mediated sexual selection in a ladybird/sexually transmitted disease system. Anim. Behav. 63:131-141

Webberley KM, Hurst GDD, Husband RW, Schulenburg J, Sloggett JJ, Isham V, Buszko J, Majerus MEN (2004) Host reproduction and a sexually transmitted disease: causes and consequences of *Coccipolipus hippodamiae* distribution on coccinellid beetles. J. Anim. Ecol. 73:1-10

Webberley KM, Tinsley MC, Sloggett JJ, Majerus MEN, Hurst GDD (2006) Spatial variation in the incidence of a sexually transmitted parasite of the ladybird beetle *Adalia bipunctata* (Coleoptera : Coccinellidae). Eur. J. Entomol. 103:793-797

Weissenberger A, Brun J, Piotte C, Ferran A (1999) Comparison between the wild type and flightless type of the coccinellid *Harmonia axyridis* (Pallas) in the control of the damson hop aphid *Phorodon humuli* (Schrank). Conference: Fifth International Conference on Pests in Agriculture pp. 727–734

Weisser WW, Braendle C, Minoretti N (1999) Predator-induced morphological shift in the pea aphid. Proc. R. Soc. Lond. B 266:1175-1181

White EB, Debach P, Garber MJ (1970) Artificial selection for genetic adaptation to temperature extremes in *Aphytis lingnanensis* Compere (Hymenoptera-Aphelinidae). Hilgardia 40:161-192

Wittkopp PJ, Beldade P (2009) Development and evolution of insect pigmentation: Genetic mechanisms and the potential consequences of pleiotropy. Sem. Cell Dev. Biol. 20:65-71

Wittkopp PJ, Carroll SB, Kopp A (2003) Evolution in black and white: genetic control of pigment patterns in *Drosophila*. Trends Genet. 19:495-504

Wratten SD (1973) Effectiveness of coccinellid beetle, *Adalia bipunctata* (L), as a predator of lime aphid, *Eucallipterus tiliae* L. J. Anim. Ecol. 42:785-802

Wratten SD (1976) Searching by *Adalia bipunctata* L (Coleoptera-Coccinellidae) and escape behavior of its aphid and cicadellid prey on lime (*Tilia* x *vulgaris* Hayne). Ecol. Entomol. 1:139-142

Wyss E (1995) The effect of weed strips on aphids and aphidophagous predators in an apple orchard Entomol. Exp. Appl. 75:43-49

Wyss E, Villiger M, Hemptinne JL, Müller-Schärer H (1999a) Effects of augmentative releases of eggs and larvae of the ladybird beetle, *Adalia bipunctata*, on the abundance of the rosy apple aphid, *Dysaphis plantaginea*, in organic apple orchards. Entomol. Exp. Appl. 90:167-173

Wyss E, Villiger M, Müller-Schärer H (1999b) The potential of three native insect predators to control the rosy apple aphid, *Dysaphis plantaginea*. BioControl 44:171-182

Yasuda H, Dixon AFG (2002) Sexual size dimorphism in the two spot ladybird beetle *Adalia bipunctata*: developmental mechanism and its consequences for mating. Ecol. Entomol. 27:493-498

Yasui H (2009) Chemical communication in mate location and recognition in the white-spotted longicorn beetle, *Anoplophora malasiaca* (Coleoptera: Cerambycidae). Appl. Entomol. Zoolog. 44:183-194

Yasui H, Akino T, Yasuda T, Fukaya M, Ono H, Wakamura S (2003) Ketone components in the contact sex pheromone of the white-spotted longicorn beetle, *Anoplophora malasiaca*, and pheromonal activity of synthetic ketones. Entomol. Exp. Appl. 107:167-176

Yasui H, Inouchi J, Wakamura S, Fukaya M, Akino T, Arakaki N (2012) Male mate searching behavior in the black chafer *Holotrichia loochooana loochooana* (Sawada) (Coleoptera: Scarabaeidae): response to pheromone lures of various colors and intensities. J. Ethol. 30:233-238

Zakharov IA, Goryacheva, II (1998) A study of nonrandom mating in the polymorphic *Adalia bipunctata* populations of the Crimea. Genetika 34:1090-1093

Zeh JA, Zeh DW (1997) The evolution of polyandry. 2. Post-copulatory defences against genetic incompatibility. Proc. R. Soc. Lond. B 264:69-75

Zera AJ, Denno RF (1997) Physiology and ecology of dispersal polymorphism in insects. Annu. Rev. Entomol. 42:207-230

Zera AJ, Harshman LG (2001) The physiology of life history trade-offs in animals. Annu. Rev. Ecol. Syst. 32:95-126

Zera AJ, Mole S (1994) The physiological costs of flight capability in wing dimorphic crickets Res. Popul. Ecol. 36:151-156

Zera AJ, Mole S, Rokke K (1994) Lipid, carbohydrate and nitrogen-content of long-winged and short-winged *Gryllus firmus*-implications for the physiological cost of flight capability. J. Ins. Phys. 40:1037-1044

Zhang XS (2008) Increase in quantitative variation after exposure to environmental stresses and/or introduction of a major mutation: *G* x *E* interaction and epistasis or canalization? Genetics 180:687-695

Zuparko RL, Dahlsten DL (1994) Host plant resistance and biological control for linden aphids. J. Arboriculture 20:278-281

Zuparko RL, Dahlsten DL (1995) Parasitoid complex of *Eucallipterus tiliae* (Homoptera, Drepanosiphidae) in Northern California. Environ. Entomol. 24:730-737

Zuparko RL, Dahlsten DL (1996) New potential for classical biological control of *Eucallipterus tiliae* (Homoptera: Drepanosiphidae). Biol. Control 6:407-408

Affiliations of all authors

	At the time of the research	*Currently*
Ellyn V. Bitume	Institute of Biology, Leiden University, The Netherlands	Evolutionary Ecology and Genetics, Université catholique de Louvain, Belgium
Paul M. Brakefield	Institute of Biology, Leiden University, The Netherlands	Zoological Department of Cambridge/ Zoological Museum of Cambridge, UK
Bardo A. Cornelder	Institute of Biology, Leiden University, The Netherlands	
Peter W. de Jong	Laboratory of Entomology, Wageningen University, The Netherlands	Laboratory of Entomology, Wageningen University, The Netherlands
Thomas C. Holness	Applied Plant Research - Flower Bulbs and Nursery Stock, Wageningen UR, The Netherlands	
Kees G. Koops	Institute of Biology, Leiden University, The Netherlands	Institute of Biology, Leiden University, The Netherlands
Suzanne T.E. Lommen	Institute of Biology, Leiden University, The Netherlands	Department of Biology, University of Fribourg, Switzerland
Carola A. Luijten	Institute of Biology, Leiden University, The Netherlands	
Cock W. Middendorp	Laboratory of Entomology, Wageningen University, The Netherlands	
Suzanne V. Saenko	Institute of Biology, Leiden University, The Netherlands	Department of Genetics & Evolution, University of Geneva, Switzerland
Yoshinori Tomoyasu	Division of Biology, Kansas State University, USA	Department of Zoology, Miami University, USA
Alfons J. van Kuik	Applied Plant Research - Flower Bulbs and Nursery Stock, Wageningen UR, The Netherlands	Applied Plant Research - Flower Bulbs and Nursery Stock, Wageningen UR, The Netherlands
Jeroen van Schelt	Koppert B.V. , The Netherlands	Koppert B.V. , The Netherlands

Nederlandse samenvatting | Summary in Dutch

Het onderzoeken en gebruiken van natuurlijke variatie in vleugellengte van een roofzuchtig lieveheersbeestje voor biologische bestrijding

Het leven op aarde is enorm divers, waarbij organismen variëren in kenmerken als uiterlijk, levenswijze en gedrag. Een centrale vraag in de evolutiebiologie is hoe en waarom er zo'n grote variatie aan levensvormen bestaat. Wanneer variatie tussen individuen van dezelfde soort voorkomt, spreekt men van intraspecifieke variatie. Binnen het tweestippelige lieveheersbeestje, *Adalia bipunctata*, komt bijvoorbeeld natuurlijke variatie in vleugellengte voor. In dit proefschrift onderzoek ik met een multidisciplinaire en experimentele aanpak hoe deze variatie wordt veroorzaakt en welke gevolgen dit heeft voor het organisme. Daarmee probeer ik te verklaren waarom deze intraspecifieke variatie voorkomt.

De mens heeft altijd geprobeerd om de natuur en diens variatie in zijn eigen voordeel te benutten. Zo wordt het tweestippelige lieveheersbeestje als rover van bladluizen ingezet bij de bestrijding van dit plaagorganisme (Afbeelding 1). Dit heeft niet altijd het gewenste effect. De neiging van gevleugelde lieveheersbeestjes om weg te vliegen van de plant wordt als één van de oorzaken gezien (Afbeelding 2). In dit proefschrift onderzoek ik daarom ook of de effectiviteit van biologische bestrijding met het tweestippelige lieveheersbeestje kan worden verbeterd door gebruik te maken van exemplaren met kortere vleugels.

Met de opgedane kennis over variatie in vleugellengte geef ik advies over de verdere ontwikkeling van een ongevleugeld tweestippelig lieveheersbeestje als commercieel product. Zo leg ik met dit proefschrift een link tussen het vakgebied van de fundamentele evolutiebiologie en de toegepaste biologische bestrijding.

Achtergrond - *Adalia bipunctata*

Adalia bipunctata is inheems in grote delen van Europa en Noord-Amerika. Een eitje ontwikkelt zich tot ongevleugelde larve, die na de uitkomst drie keer vervelt voordat hij zich verpopt, en zich tenslotte tot volwassen gevleugeld kevermannetje of -vrouwtje ontpopt (Afbeelding 3A-E). Na een succesvolle paring kan een vrouwtje bijna haar hele leven eitjes blijven leggen en dat doet ze op planten met bladluizen. Omdat bladluizenpopulaties door de tijd heen sterk fluctueren in grootte en locatie, vliegen de volwassen lieveheersbeestjes van de ene waardplant naar de andere.

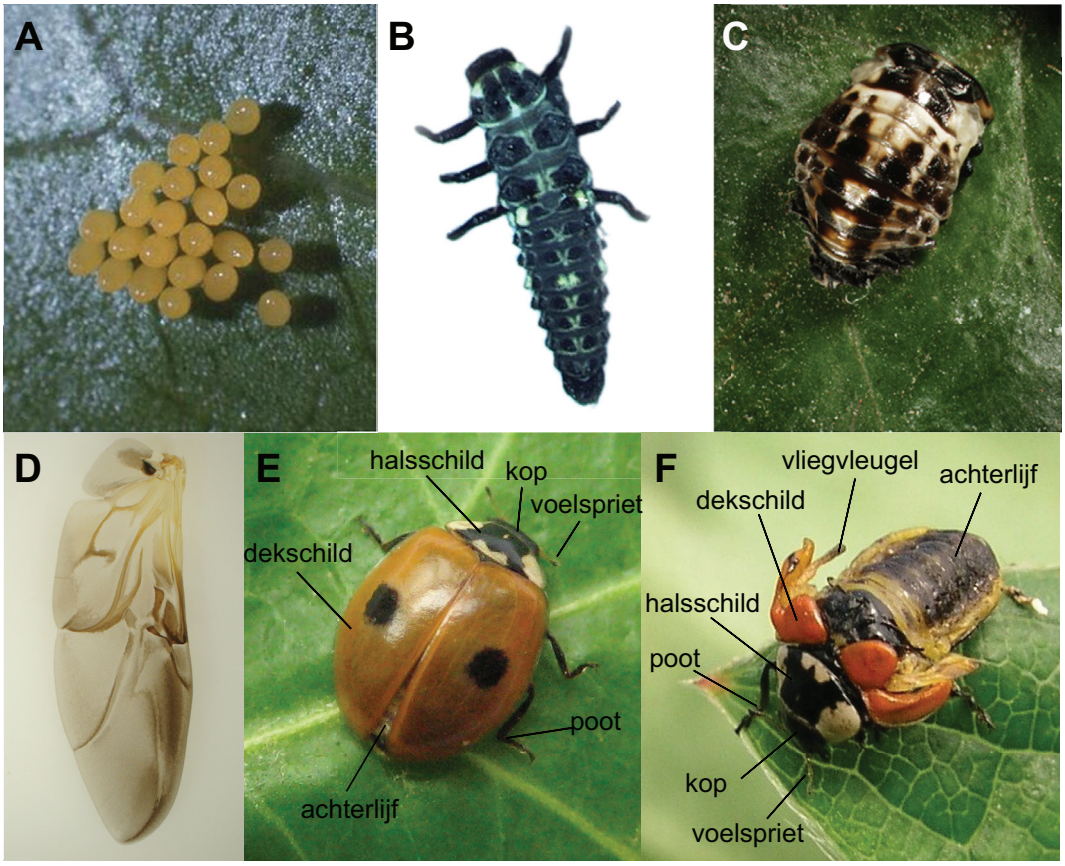
Afbeelding 1. Een zevenstippelig lieveheersbeestje eet een bladluis (foto: Hans Smid, www.bugsinthepicture.com)

Afbeelding 2. Een tweestippelig lieveheersbeestje vliegt op (foto: Kjeld Olesen, www.acapixus.dk)

Zowel de larven als de volwassen lieveheersbeestjes leven voornamelijk van bladluizen en daar wordt gebruik van gemaakt in de landbouw: het tweestippelige lieveheersbeestje wordt massaal gekweekt voor de biologische bestrijding van bladluizen, waarbij de larven of volwassen kevers worden uitgezet in het aangetaste gewas. Deze vorm van biologische bestrijding is echter niet altijd effectief. Een van de problemen is dat de volwassen lieveheersbeestjes snel wegvliegen uit het gewas (Afbeelding 2).

In de natuur komt echter een 'ongevleugelde' variant van het tweestippelige lieveheersbeestje voor (Afbeelding 3F). Deze variant is zeldzaam, maar in tenminste één Nederlandse en vijf Engelse populaties zijn zulke exemplaren waargenomen (respectievelijk door de groep van Paul Brakefield en Michael Majerus). Een 'ongevleugelde' volwassene heeft afgeknotte vliegvleugels én afgeknotte dekschildjes (de dekschildjes waren oorspronkelijk ook vliegvleugels, maar zijn in de evolutie van kevers veranderd in beschermende schilden). Deze misvorming is erfelijk bepaald en wordt gereguleerd door een ondergeschikte variant van het corresponderende gen (stuk DNA). Alleen individuen met twee kopieën van deze variant zijn daarom daadwerkelijk ongevleugeld.

Voor het onderzoek zijn lieveheersbeestjes verzameld in Utrecht en verder gekweekt in ons laboratorium (Afbeelding 4-6). In deze verzameling vonden we in totaal drie kopieën van het DNA dat correspondeert met de ongevleugelde variant. Door kruisingen tussen lieveheersbeestjes met en zonder dit kenmerk hebben we een pure ongevleugelde en een pure gevleugelde ('wildtype') kweeklijn gekregen. Deze vormden samen het studiemateriaal voor dit onderzoek.



Afbeelding 3. De levenscyclus van het tweestippelige lieveheersbeestje: van ei (A), via larve (B) en pop (C) tot volwassen kever, gevleugeld (E, vliegvleugel in D) of ongevleugeld (F)

Deel A - de effectiviteit van ongevleugelde *Adalia bipunctata* als bladluisbestrijder

Ik laat eerst zien zien dat **het gebruik van ongevleugelde lieveheersbeestjes de bestrijding van bladluizen in principe kan verbeteren** (hoofdstuk 2). Daarvoor hebben we paprikaplanten met bladluizen besmet en daar vervolgens een gevleugeld danwel ongevleugeld lieveheersbeestje op los gelaten. Na twee dagen heb ik het resultaat vergeleken met planten zonder lieveheersbeestje. Daaruit blijkt dat ongevleugelde lieveheersbeestjes inderdaad langer op de planten blijven dan gevleugelde en dat ze de groei van de bladluispopulatie kunnen remmen ten opzichte van onbehandelde planten. Hun meerwaarde ten opzichte van de gevleugelden hangt echter af van de soort bladluis: bij de groene perzikluis (*Myzus persicae*) waren ongevleugelde lieveheersbeestjes effectiever, maar bij de boterbloemluis (*Aulocorthun solani*) hadden de gevleugelde lieveheersbeestjes in kortere tijd net zoveel effect. We tonen aan dat dit niet wordt verklaard door een verschil in eetgedrag

tussen de lieveheersbeestjes en suggereren dat dit wel verklaard zou kunnen worden door een verschil in verdedigingsgedrag tussen de beide bladluissoorten. We verwachten echter dat dit een korte-termijn effect is en dat ongevleugelde lieveheersbeestjes op langere termijn in het algemeen effectiever zijn in bladluisbestrijding in kassen dan gevleugelde lieveheersbeestjes.

Vervolgens toon ik aan dat **het uitzetten van ongevleugelde lieveheersbeestjes in lindebomen in de stad de honingdauw onder deze bomen kan verminderen** (hoofdstuk 3). Lindes komen veel voor in steden en worden jaarlijks gekoloniseerd door lindebladluizen, die grote hoeveelheden honingdauw kunnen produceren. Deze suikeroplossing drupt van de bomen en vormt een kleverige laag op objecten daaronder, zoals stoepen, auto's en bankjes. Door wekelijks larven of volwassen exemplaren van de ongevleugelde kweeklijn uit te zetten op de stam van de bomen, verminderde de honingdauw onder de Hollandse linde (*Tilia x vulgaris*) en de winterlinde (*Tilia cordata*) in het Amsterdamse stadsdeel Slotervaart aanzienlijk. Hiermee laten we zien dat het buiten uitzetten van ongevleugelde tweestippelige lieveheersbeestjes ook potentie heeft. In vervolgonderzoek moet worden aangetoond of ze ook in die situatie effectiever zijn dan gevleugelde varianten en of het uitzetten van ongevleugelde lieveheersbeestjes een kosteneffectieve maatregel is.

Afbeelding 4. Aan het werk in de kweekkamer (foto: René Glas, www.reneglas.com)

Afbeelding 5. Lieveheersbeestjes in een vierkante Petrischaal, met erboven een zachte pincet als onmisbaar instrument voor de onderzoeker, en pollen en motteneieren als kevervoer

Afbeelding 6. Schalen vol lieveheersbeestjes in de kweekkamer

Deel B - Oorzaak en gevolg van variatie in vleugellengte in *Adalia bipunctata*

Het wordt pas interessant om ongevleugelde varianten van het tweestippelige lieveheersbeestje als biologische bestrijders op de markt te brengen wanneer ze aan twee voorwaarden voldoen: ze moeten niet alleen effectiever zijn in bladluisbestrijding dan gevleugelde varianten maar moeten ook kosten-effectief te kweken zijn. Dat laatste was tot nu toe een zorg, omdat Hideki Ueno had laten zien dat de ongevleugelde varianten gemiddeld minder 'fit' waren dan gevleugelde: de larven ontwikkelden zich langzamer, de volwassenen lieveheersbeestjes leefden korter en de vrouwtjes produceerden daardoor ook minder nakomelingen.

In laboratoriumkweken van ongevleugelde lieveheersbeestjes bleek echter dat ze **onderling sterk variëren in de mate van vleugelreductie**: hoewel ze allen genetisch gezien ongevleugeld zijn, varieert het uiterlijk via een continue reeks van individuen die helemaal geen vleugelweefsel meer hebben tot individuen die bijna geen vleugelweefsel missen en dus in uiterlijk nauwelijks afwijken van het wildtype (Afbeelding 7). Hideki Ueno vond aanwijzingen dat deze variatie gedeeltelijk erfelijk werd bepaald én dat er een relatie bestond tussen de mate van vleugelreductie en de eerder genoemde levensloopkenmerken: hoe langer de vleugels, hoe beter de ongevleugelde lieveheersbeestjes presteerden. Ik heb het onderzoek naar de oorzaken en gevolgen van deze variatie voortgezet.

Eerst verdiep ik me in de genetica. Per toeval ontdekken we dat **het kenmerk 'ongevleugeldheid' samenhangt met de genetische variatie in kleur** (hoofdstuk 4). De vele kleurvarianten van het tweestippelige lieveheersbeestje zijn grofweg in te delen in een groep van rode dekschilden met zwarte stippen en zwarte halsschilden met witte vlekken ('typica') en een groep van zwarte dekschilden met rode stippen en zwarte halsschilden zonder witte vlekken ('melanic') (Afbeelding 7). De kleur bleek

Afbeelding 7. Ongevleugelde tweestippelige lieveheersbeestjes variëren in de mate van vleugelreductie en in kleur: ´typica´ en ´melanic´ (de twee middelste exemplaren)

vaak samen over te erven met het ongevleugelde kenmerk. Dit gegeven kan het identificeren van de betreffende verantwoordelijke genen vergemakkelijken.

Dan presenteer ik **een model voor de onderliggende genetica van de variatie in vleugelreductie binnen de ongevleugelde lieveheersbeestjes** (hoofdstuk 5). Door families te kweken die verschillen in de mate van vleugelreductie, bevestig ik Hideki's waarneming dat deze variatie een erfelijke component bezit. Op basis van een stamboom concludeer ik dat daar tenminste twee andere genen aan ten grondslag moeten liggen dan het gen voor ongevleugeldheid zelf. Ik bewijs echter dat de omgevingstemperatuur waarbij de larven worden gekweekt ook mee bepaalt hoe sterk de uiteindelijke vleugelreductie in de volwassenen is. Vervolgens demonstreer ik dat **de mate van vleugelreductie van een ongevleugelde kweeklijn te manipuleren is** door individuen met de gewenste vleugellengte te kiezen als ouders van de volgende generatie ('kunstmatige selectie'). Na vier generaties heb ik enerzijds kweeklijnen geproduceerd waarvan de meeste ongevleugelden maar een klein puntje van hun vleugels missen en anderzijds kweeklijnen waarvan de meeste lieveheersbeestjes juist helemaal geen vleugelweefsel meer hebben. De eerstgenoemden planten zich beter voort dan de tweede, terwijl ze nog steeds niet of nauwelijks kunnen vliegen. Daarom stel ik dat deze geselecteerde lijnen met milde vleugelreductie veel potentie hebben om de massakweek van ongevleugelde lieveheersbeestjes te verbeteren.

Deze bevindingen leiden ook tot een algemene beschouwing van de evolutie van vleugelverlies in insecten. Veel insectensoorten hebben in de evolutie hun vleugels (gedeeltelijk) verloren. Eén theorie stelt dat dit stapsgewijs is verlopen, door een geleidelijke steeds verdere verkleining van de vleugels, maar hier is weinig bewijs voor. Ik stel dat de kunstmatige evolutie in vleugellengte die we hebben bewerkstelligd in het tweestippelig lieveheersbeestje laat zien dat zo'n mechanisme van graduele evolutie in principe mogelijk is.

Dan bestudeer ik de vleugelontwikkeling van de lieveheersbeestjes (hoofdstuk 6). Ik toon aan dat de **vleugelreductie in volwassen ongevleugelde lieveheersbeestjes het gevolg is van een vertraagde ontwikkeling van het vleugelweefsel in de larven**. Deze vertraging blijkt samen te hangen met

een veranderde expressie van het gen *Distal-less.* Het is bekend dat dit gen in het modelinsect, het fruitvliegje *Drosophila melanogaster*, een belangrijke rol vervult in de vleugelontwikkeling. Deze kennis biedt verdere aanknopingspunten voor de toekomstige identificatie van het gen dat ongevleugeldheid in het tweestippelige lieveheersbeestje reguleert.
Tenslotte laat ik met behulp van gedragsobservaties zien dat **reductie in vleugellengte van vrouwtjes een negatief effect heeft op het paargedrag van mannetjes** (hoofdstuk 7). Wanneer een mannetje slechts een enkel vrouwtje kreeg aangeboden, was de kans dat zij gepaard werd groter naarmate haar vleugels langer waren. Als een mannetje echter de keus had tussen een gevleugeld en een ongevleugeld vrouwtje, paarde hij steevast met het gevleugelde vrouwtje. We suggereren dat de fysieke aanwezigheid van de vrouwekijke dekschildjes de paring faciliteert door het mannetje houvast te bieden.

Deel C - synthese en perspectief

In de synthese (hoofdstuk 8) beschouw ik bovenstaande resultaten eerst in het licht van evolutie en daarna van biologische bestrijding. Ik geef een samenvatting van alle tot nu toe gevonden verschillen tussen gevleugelde en ongevleugelde varianten van het tweestippelige lieveheersbeestje in kenmerken die belangrijk zijn voor overleving en reproductie. Wat betreft de meeste kenmerken is de ongevleugelde variant sterk in het nadeel. Op basis van deze kennis is te verwachten dat de natuur tegen ongevleugeldheid selecteert, zodat het kenmerk uiteindelijk uit populaties verdwijnt. Met andere woorden: **het is onwaarschijnlijk dat ongevleugeldheid in het tweestippelige lieveheersbeestje voorkomt als evolutionaire aanpassing**. Het is plausibeler dat dit kenmerk in stand wordt gehouden door toevalligheid of door een evenwicht tussen de mutatiesnelheid (het kenmerk ontstaat door natuurlijke genetische mutatie) en natuurlijke selectie (die tegen het kenmerk selecteert).
Uit het onderzoek naar de biologische bestrijding van bladluizen concludeer ik dat **de ongevleugelde variant van het tweestippelig lieveheersbeestje goed perspectief biedt voor verbetering van de huidige bestrijding met gevleugelde varianten**. Ik adviseer om **gebruik te maken van ongevleugelden die zijn geselecteerd voor milde vleugelreducties**, omdat die naar verwachting beter te kweken zijn en wellicht ook nog beter presteren in bestrijding dan varianten met sterke reductie. Verder onderzoek zal moeten uitwijzen of dit in praktijk haalbaar en kosteneffectief is.
Tenslotte breek ik in een algemeen perspectief (hoofdstuk 9) een lans voor **het vaker toepassen van kunstmatige selectie voor de verbetering van natuurlijke vijanden voor biologische bestrijding**. Ik denk dat er op die manier beter gebruik kan worden gemaakt van genetische natuurlijke variatie. Hiermee kan een kwalitatieve verbetering worden bereikt en deze methode kan ook een strategie zijn om risico's van alternatieven met exotische organismen en genetische manipulatie te omzeilen.

Curriculum vitae

I, Suzanne Theresia Esther Lommen, was born on October 13, 1979 in Tilburg, The Netherlands. My secondary school program (Voorbereidend Wetenschappelijk Onderwijs - Gymnasium) started at the Odulphus Lyceum in Tilburg in 1991, which I completed in 1997.

I had become so enthusiastic about biology, that I started my studies in Biology later that year at Wageningen University (at that time still named Wageningen Agricultural University), The Netherlands. After two years of study, I took a year break from life sciences to become a full-time member of the board of the Wageningen Student Union, where I learnt a lot about human organisations and politics. I then continued my studies, and performed research thesis projects around the world: at the Netherlands Institute of Ecology in Heteren, The Netherlands, with Felix Wäckers; at the Zoological Institute of Copenhagen, Denmark, with Sylvia Cremer; and the Universiti Putra Malaysia, Malaysia, with Ahmad Sajap. The general theme that these projects had in common was the ecology and evolution of insect species, and specifically their interactions with different species of organisms. I strengthened my communication skills by working part time as a journalist for the Wageningen University Newspaper, and as an assistant in the Department of Educational Studies. I obtained my Master degree (Ingenieursdiploma) in Biology, with specializations in Population Biology and Ecology, with distinction (cum laude), in 2003.

I then continued the scientific study of insects in the Evolutionary Biology Lab of Paul Brakefield at the Institute of Biology, Leiden University, The Netherlands. From 2004 to 2008, I worked on my PhD research 'Exploring and exploiting natural variation in the wings of a predatory ladybird beetle for biological control'. Peter de Jong from Wageningen University was my co-supervisor. I collaborated with Yoshi Tomoyasu from Kansas State University, USA, with the Dutch institute of Applied Plant Research of Wageningen UR, and the Dutch company Koppert B.V., and presented my work at several international scientific meetings. I gained more experience in organisation as a member of the Committee of Scientific Meetings of the institute and by co-organising the PhD course 'Writing a Grant Proposal'.

In 2009, I moved to the institute of Applied Plant Research of Wageningen UR in Lisse, The Netherlands, to work as an entomological researcher on crop protection. I dealt with insect pests, mite pests and plant pathogens, and learnt how to manage research projects, to transfer knowledge, and to engage stakeholders in research.

Since 2013 I am employed as a Postdoctoral Researcher at the Plant Population Group of Heinz Müller-Schärer at the University of Fribourg, Switzerland. I examine the potential of the biological control of the invasive allergenic ragweed*, Ambrosia artemisiifolia* by insect herbivores. This is performed within the framework of SMARTER, a European interdisciplinary research network on ragweed control that has recently been set up. I also play a coordinative role in this network.

Publications

Papers related to this thesis and published in peer-reviewed international scientific journals

Lommen STE, Holness TC, De Jong PW, Van Kuik AJ, Brakefield PM (2013). Releases of a natural flightless strain of the ladybird beetle *Adalia bipunctata* reduce aphid-born honeydew beneath urban lime trees. **BioControl** 58: 195-204 [CHAPTER 3]

Lommen STE, De Jong PW, Koops KG, Brakefield PM (2012). Genetic linkage between melanism and winglessness in the ladybird beetle *Adalia bipunctata*. **Genetica** 140: 229-233 [CHAPTER 4]

Lommen STE, Saenko SV, Tomoyasu Y, Brakefield PM (2009). Development of a wingless morph in the ladybird beetle*, Adalia bipunctata*. **Evolution & Development** 11: 278-289 [CHAPTER 6]

Lommen STE, Middendorp CW, Luijten CA, Van Schelt J, Brakefield PM, De Jong PW (2008). Natural flightless morphs of the ladybird beetle *Adalia bipunctata* improve biological control of aphids on single plants. **Biological Control** 47: 340-346 [CHAPTER 2]

Lommen STE, Koops K, De Jong PW, Brakefield PM (2005). Phenotypic plasticity of elytron length in wingless two-spot ladybird beetles. **European Journal of Entomology** 102: 553-556

Other published papers related to this thesis

Lommen S, Van Kuik F (2012). Ongevleugelde lieveheersbeestjes. Oplossing voor honingdauw onder lindebomen? **Bomen** 21: 14-17 [in Dutch]

Lommen STE, Middendorp CW, Luijten CA, Van Schelt J, Brakefield PM, De Jong PW (2008). Can natural flightless ladybirds improve biocontrol of aphids? **IOBC-WPRS Bulletin** 32, 127-130 [Proceedings of a meeting of the Working Group 'Integrated Control in Protected Crops, Temperate Climate', Sint-Michielsgestel, The Netherlands 2008]

Lommen STE, Cuppen JGM (2007). Ladybird beetles: rulers of the fields? **Entomologische Berichten** 67: 260-263 [In Dutch, special issue on agrobiodiversity]

Other published papers

Lommen STE, Conijn CGM, Lemmers MEC, Pham KTK, De Kock MJD (2012). Mites as vector of *Tulip Virus X* in stored tulip bulbs. **IOBC-WPRS Bulletin** 81: 57-67 [Proceedings of a meeting of the Working Group 'Integrated Protection of Stored Products', Volos, Greece 2011]

Cremer S, Ugelvig LV, Lommen STE, Petersen KS, Pedersen JS (2006). Attack of the invasive garden ant: aggression behaviour of *Lasius neglectus* (Hymenoptera: Formicidae) against native *Lasius* species in Spain. **Myrmecologische Nachrichten** (now: Myrmelogical News) 9: 13-19

Lommen STE, Van Huis A, Sajap AS (2004). Caste biology and behaviour of *Bulbitermes sarawakensis* (Isoptera, Nasutitermitinae) in Malaysia. **Proceedings of the Netherlands Entomological Society Meeting** 15: 21-28

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